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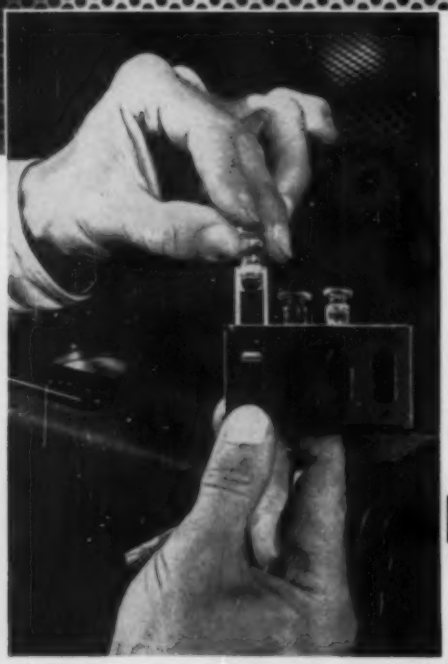

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVII — *AUGUST 1958* — NUMBER 8

Antibacterial and Antifungal Properties of β-Naphthol Derivatives V*

By R. S. BAICHWAL,† MALATI R. BAICHWAL,† and M. L. KHORANA‡

A detailed study was made on some derivatives of β-naphthol which have emerged as active antibacterial and antifungal compounds from a preliminary screening already reported. This included the evaluation of penetration power, influence of organic matter, and fungicidal activity. Further tests were carried out on a selected group of compounds, and covered such determinations as topical irritation and interference in the process of wound healing. The LD_{50} by intraperitoneal administration of 6-hexyl-β-naphthol was found to be 127 mg. per Kg. A potentiation of fungistatic action was observed with a few naphthol derivatives in formulations containing known anti-infective agents. All compounds seemed to possess good penetration power even in ointment vehicles and were nonirritant to the intact skin of rabbits. The presence of organic matter did not appear to curtail the activities of these compounds seriously and the compounds themselves did not seem to have any adverse effect on the process of wound healing. The results obtained from these limited tests indicated that the compounds may prove useful as anti-infective agents and hence seem worthy of further toxicity and clinical studies.

PRELIMINARY SCREENING of a large number of derivatives of β-naphthol for antibacterial and antifungal activities showed some compounds to exhibit preferential activity against Gram-positive bacteria and human pathogenic fungi (1). The results suggested further studies on their anti-infective properties under a variety of test conditions simulating practical conditions of use. The selected compounds were evaluated as to methods of sterilization, penetration power, influence of organic matter, irritation on intact skin, interference in healing of wounds, anti-inflammatory property, and acute toxicity. Further, the antifungal compounds were tested for their fungicidal activity, and a few formulations, containing the selected compounds, for their fungistatic action.

* Received February 3, 1958, from the Bombay University Department of Chemical Technology, Bombay 19, India.

This paper is based in part on the theses submitted to the University of Bombay by R. S. Baichwal and Malati R. Baichwal for the degree of Doctor of Philosophy in the Faculty of Technology.

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‡ Reader in Pharmaceutical Chemistry, Bombay University Department of Chemical Technology, Bombay 19, India.

EXPERIMENTAL

Methods of Sterilization.—Tests were conducted to determine whether the active antibacterial compounds could withstand moist heat sterilization without impairment of their bacteriostatic activity.

Nutrient broth containing the previously determined (1) inhibitory concentrations of the test substances were autoclaved at 15 lb. pressure for twenty minutes. Results observed following inoculation of a loopful of a twenty-four hour culture of the test organisms and incubation for twenty-four to forty-eight hours are recorded in Table I. All the bromoanilides were found to lose their activity by this treatment. However, they retained their inhibitory power after being subjected to dry heat sterilization at 150° for one hour.

Penetration Power.—The agar cup-plate method (2) was followed using *M. pyogenes* var. *aureus* and var. *albus* as the test organisms. One cubic centi-

TABLE I.—BACTERIOSTATIC ACTIVITY OF β-NAPHTHOL DERIVATIVES AFTER MOIST HEAT STERILIZATION[a]

No.	Compound	M. p., °C.	*M. pyogenes* var. *aureus* Concentration in μg./cc. 10	5	*M. pyogenes* var. *albus* Concentration in μg./cc. 10	5
1	3:6-Dibromo-β-naphthol	127	–[c]	–	–	–
2	1:3:6-Tribromo-β-naphthol	132	–	–	–	+[d]
3	3:4:6-Tribromo-β-naphthol	128	–	–	–	–
4	6:6′-Dibromo-β-dinaphthol	202	–	–	–	+
5	6-*n*-Pentyl-β-naphthol	103.5	–	–	–	–
6	6-*n*-Hexyl-β-naphthol	101.5	–	–	–	–
7[b]	1-Bromo-2-hydroxy-3-naphthanilide	162	+	+	+	+
8[b]	1:4′-Dibromo-2-hydroxy-3-naphthanilide	234	+	+	+	+
9[b]	1:6-Dibromo-2-hydroxy-3-naphthanilide	182	+	+	+	+
10[b]	1:4′:6-Tribromo-2-hydroxy-3-naphthanilide	218	+	+	+	+

[a] Fifteen lbs. pressure for twenty minutes.
[b] Retained bacteriostatic activity after dry sterilization at 150° for one hour.
[c] –, no growth. [d] +, growth.

meter of a twenty-four hour culture was added to 9 cc. of nutrient agar broth and poured into Petri plates containing 20 cc. of agar. Fine suspensions of the test compounds in concentrations of 50, 20, and 10 μg./cc. in 5% gum acacia were added to 10-mm. cups previously prepared. Diameters of zones of complete inhibition were measured after incubation for forty-eight hours. Table II records the zone diameters shown at a concentration of 50 μg./cc. (column I), and the difference in zone diameters between those at 50 μg./cc. and 10 μg./cc. concentrations (column II).

The penetrability of the more active compounds (Table II), such as 6-*n*-hexyl-β-naphthol, 1:4′-dibromo-, and 1:6-dibromo-2-hydroxy-3-naphthanilides, was evaluated in ointment vehicles using the base recommended for sterilized cream of penicillin, B. P. 1948, by the agar-plate method (3). Using *M. pyogenes* var. *aureus*, the zones of complete inhibition compared favorably with those of acriflavine run under similar conditions, and were greater than those exhibited by the parent compound, β-naphthol.

For comparison purposes, antifungal compounds selected from the preliminary screening (1) were also tested under similar conditions (3) using *Trichophyton gypseum* as the test organism. The tests revealed that 8-hydroxyquinoline was the best and the other compounds in decreasing order of activity were, 1-bromo-, 6-bromo-, 1:6-dinitro-β-naphthols, salicylanilide, and undecylenic acid.

TABLE II.—PENETRATION POWER OF β-NAPHTHOL DERIVATIVES AT A CONCENTRATION OF 50 μG./CC.

Compound[a]	*M. pyogenes* var. *aureus* I[b]	II[c]	*M. pyogenes* var. *albus* I[b]	II[c]
1	14	4	15	4
2	13	2.5	13	3
3	12	2	12	2
4	10	0	10	0
5	10[d]	0	13[d]	2.5
6	10[d]	0	12[d]	2
7	17[d]	4	16[d]	5
8	17	2.5	18	3.5
9	25	8	20	6
10	16	2	21	5

[a] Numbers refer to compounds listed in Table I.
[b] Average diameter in mm. of the zones of complete inhibition.
[c] Difference between zone diameter at 50 μg./cc. and at 10 μg./cc.
[d] Considerable zones of diffused growth were observed.

Influence of Organic Matter.—The bacteriostatic concentrations of the selected compounds (1) were determined using twenty-four hour cultures of *M. pyogenes* var. *aureus* and var. *albus* in varying amounts such as 0.02 cc. to 0.2 cc. (viable count, 382 million/cc.). The inhibitory concentrations were found to be the same as determined previously (1) which indicated that the presence of heavy inocolum had no deleterious effect.

Tests using horse serum as the source of organic matter were conducted with the most promising compound, 6-hexylnaphthol, on lines similar to those used for hexachlorophene (4). From the results recorded in Table III, it appears that, although the presence of serum lowers the antibacterial activity, it is only over short durations. However, when long time contact is permitted, then it appears that the presence of serum is not too detrimental. Further, the loss of activity due to presence of serum could be overcome by increasing the concentration.

The antifungal compounds selected from the preliminary screening (1) were evaluated using the serum agar-cup-plate (2) as well as the agar streak method (5). The former method was similar to that described previously (1) excepting for the addition of 10% serum to the agar medium. From the graphs shown in Fig. 1 of each substance with the log concentration *vs.* average diameter of the zone of inhibition, comparison of activities can be made. For example, 6-bromo- and 1:6-dinitro-β-naphthols seem to be superior to 8-hydroxyquinoline until a concentration of 0.25% is reached. However, when compared with salicylanilide or undecylenic acid, the naphthol derivatives are found to show greater zone diameters over the entire range of concentration of 0.01 to 5%.

Following the method of Oster and Golden (6) but using a clearance zone of 3 mm. with a cup of 10 mm. and allowing a preliminary incubation of the test organism for six hours before the addition of the test substances, as followed in these tests, the activity coefficients calculated are as follows: undecylenic acid 0.4, salicylanilide 10, 8-hydroxyquinoline 20, 1-bromonaphthol 40, 6-bromonaphthol 40, and 1:6-dinitronaphthol 100. Apparently, the β-naphthol derivatives seem to be superior to the commonly used antifungal agents under the test conditions followed.

TABLE III.—EFFECT OF ORGANIC MATTER ON THE ANTIBACTERIAL ACTIVITY OF 6-*n*-HEXYL-β-NAPHTHOL

Amount Tested	Results[a] after Contact with *M. pyogenes* var. *aureus* for: 10 Min.	3 Hr.	24 Hr.	48 Hr.
Hexyl-β-naphthol				
1:1,000 in Serum 50%	...[b]	1,200	0	0
1:1,000 in Serum 10%	1,000	50	0	0
1:1,000 in Serum 0%	100	0	0	0
Hexyl-β-naphthol				
1:2,000 in Serum 50%	2,000	1,500	10	0
1:2,000 in Serum 0%	250	100	0	0

[a] Test procedure: mixture allowed to stand at 37° for times noted, after which a standard loopful was transferred to nutrient agar, incubated forty-eight hours, and colonies present counted.
[b] Too many to count.

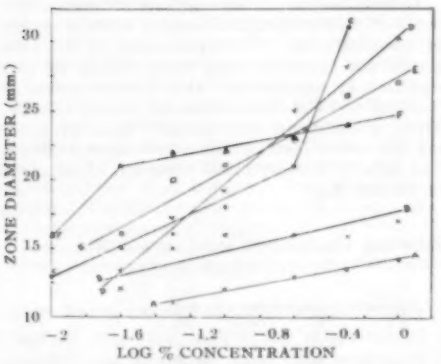

Fig. 1.—Dosage and fungistatic response curves of *T. gypseum* with increasing concentrations of β-naphthol derivatives and three commonly used antifungal agents. A–A Undecylenic acid, B–B Salicylanilide, C–C 8-Hydroxyquinoline, D–D 1-Bromo-β-naphthol, E–E 6-Bromo-β-naphthol, and F–F 1:6-Dinitro-β-naphthol.

Results obtained using the agar streak method (5) in which the agar medium contained 10% horse serum, are recorded in Table IV. The activity of 8-hydroxyquinoline against all the fungus pathogens seems to remain unaffected in the presence of serum. With the naphthol derivatives this is true only when their activities are considered against *Trichophyton rubrum* and *Epidermophyton floccosum*. However, these compounds which were inhibitory against the same organisms at a concentration of 10 μg./cc. in the absence of serum (1) were found to be ineffective at the same concentration in the presence of serum.

Fungicidal Tests.—Oster and Golden's method (6) was followed with *T. gypseum* as the test organism and 1% concentration of the test substances. Results after incubation for twenty-one days indicated that 1-bromo- and 1:6-dinitro-β-naphthols were not fungicidal even with a contact period of ten minutes. Other antifungal agents, such as undecylenic acid, salicylanilide or 8-hydroxyquinoline, which were included for comparison purposes, behaved similarly. 6-Bromo-β-naphthol was the only compound which was fungicidal in these tests. Since the compounds were tested at only one concentration, detailed comparisons of activities could not be made.

Irritation Tests.—Ointments containing the test substances in various concentrations were rubbed every day for six days on marked depilated areas on the backs of three healthy rabbits with the ointment base as the control. The ointments were prepared according to the method described under penetration tests. The results indicated that 1:6-dibromo- and 1:4′-dibromo-2-hydroxy-3-naphthanilides were nonirritant even at a concentration of 5%, while 6-hexyl-β-naphthol showed some signs of irritation.

Similar tests conducted with the antifungal agents revealed that undecylenic acid 5%, 8-hydroxyquinoline 1%, 1-bromo- and 6-bromo-naphthols 2%, and 1:6-dinitronaphthol 1% were all nonirritant. Salicylanilide 5%, however, was found to be irritant. Before any definite conclusions are drawn, it would be worth while to test these compounds again on a larger number of animals.

An attempt was next made to study irritation of the bacteriostatic compounds by the "granuloma pouch" test of Hans Selye (7, 8). In the preliminary tests, sterile arachis oil, croton oil, or 6-hexyl-β-naphthol 1% in arachis oil were injected into the prepared pouches (7, 8) on different groups of rats. The animals were sacrificed after seven days, and examination of the pouches indicated that only croton oil was irritant and not the compound or the base oil.

With a view to examining whether the test substances possessed any anti-inflammatory action, the "granuloma pouch" technique (7, 8) was repeated by administering the drugs to different groups of rats, each containing five rats, as shown in Table V. It was observed that the two compounds, 1:6-dibromo-2-hydroxy-3-naphthanilide and 6-hexyl-β-naphthol showed no anti-inflammatory action.

TABLE IV.—FUNGISTATIC ACTIVITY OF β-NAPHTHOL DERIVATIVES AT A CONCENTRATION OF 50 μG./CC. BY AGAR-STREAK METHOD

No.	Compound	*Trichophyton gypseum* Serum Absent	*Trichophyton gypseum* Serum Present	*Trichophyton rubrum* Serum Absent	*Trichophyton rubrum* Serum Present	*Epidermophyton floccosum* Serum Absent	*Epidermophyton floccosum* Serum Present
1	Undecylenic acid	+[a]	+	+	+	+	+
2	Salicylanilide	−[b]	+	−	−	−	+
3	8-Hydroxyquinoline	−	−	−	−	−	−
4	1-Bromo-β-naphthol	−	+	−	−	−	+
5	6-Bromo-β-naphthol	−	+	−	−	−	−
6	1:6-Dinitro-β-naphthol	−	+	−	−	−	−

[a] +, growth. [b] −, no growth.

TABLE V.—ANTI-INFLAMMATORY ACTIVITY OF β-NAPHTHOL DERIVATIVES

Groups	Treatment	Quantity Injected, ml.	Exudate, ml., After Ten Days
I	Arachis oil	0.5	1.2
II	1% Croton oil[a]	0.5	13 ± 1.5
III	1% Croton oil[a] and 0.5% 6-*n*-hexyl-β-naphthol[a]	0.5[b]	...
IV	1% Croton oil[a] and 0.5% 1:6-dibromo-2-hydroxy-3-naphthanilide[a]	0.5[b]	...

[a] Dissolved in sterile arachis oil.
[b] Same quantity injected again after three days.

However, they did not seem to contribute any added irritation due to their presence.

Wound Healing Tests.—For this purpose, Carrell and Hartmann's technique (9) used by Dodd and coworkers (10) was followed by using healthy rats instead of rabbits. Three male rats of average body weight of 125 Gm. were depilated, allowed to rest for twenty-four hours, anesthetized, and two excisions made on opposite sides of the dorsal midline. One of the wounds was treated with an ointment containing the test substance and the other with the base serving as control. The rest of the procedure was similar to that described by Dodd, *et al.* (10). From the results recorded in Table VI, it is apparent that 6-hexyl-β-naphthol and 1:6-dibromo-2-hydroxy-3-naphthanilide do not interfere with the process of epithelization in concentrations of 1% and 5%. However, wounds treated with 1:4′-dibromo-2-hydroxy-3-naphthanilide, even at 1% concentration require two days more for healing than the control wounds.

In case of antifungal compounds, it was observed that, with all the compounds tested, the days required for the healing of the test and control wounds were the same. Salicylanilide and 6-bromonaphthol which showed some signs of irritation on intact skin of rabbits when applied in the form of 5% ointments, had no adverse effect on the process of wound healing (Table VI).

Toxicity Tests.—An acute toxicity test was carried out with 6-hexyl-β-naphthol using different routes of administration. A stock solution of the substance was prepared using 0.1% Tween 80 and sterilized by autoclaving. Oral administration in doses up to 1 Gm./Kg. showed no toxicity to mice. When administered intravenously, doses up to 40 mg./Kg. were found to have no harmful effects. The LD_{50} by intraperitoneal route was of the order of 127 mg./Kg.

TABLE VI.—DATA ON WOUNDS TREATED WITH β-NAPHTHOL DERIVATIVES AND CONTROL WOUNDS

Substance Applied	Concn. % in Ointment	Av. Area in Sq. Cm. of Unhealed Wounds After —— days 2	3	4	5	6	7	8	9	10	11	12
6[a]	1 T	0.96	...	1.03	...	0.86	...	0.55	...	0.23	...	0.06[c]
	C	1.23	...	1.33	...	1.00	...	0.60	...	0.26	...	0.06[c]
	5 T	1.10	...	1.20	...	0.76	...	0.50	...	0.50	...	0.23[e]
	C	1.03	...	1.16	...	0.76	...	0.60	...	0.56	...	0.26[e]
8[a]	1 T	1.10	...	0.73	...	0.56	...	0.50	...	0.40	...	0.16[e]
	C	0.93	...	0.70	...	0.56	...	0.36	...	0.10	...	0
	5 T	1.0	...	0.63	...	0.53	...	0.43	...	0.33	...	0.16[e]
	C	1.03	...	0.76	...	0.60	...	0.30	...	0.08	...	0
9[a]	1 T	1.73	...	1.16	...	0.46	...	0.26	...	0.08	...	0
	C	1.60	...	1.16	...	0.50	...	0.23	...	0.08	...	0
	5 T	1.96	...	1.00	...	0.60	...	0.33	...	0.10	...	0
	C	2.00	...	1.30	...	0.63	...	0.43	...	0.20	...	0
1[b]	5 T	...	0.40	...	0.16	...	0.13	...	...	0	...	...
	C	...	0.25	...	0.20	...	0.10	...	...	0	...	...
	2 T	...	0.46	...	0.33	...	0.20	...	...	0.03	0	...
	C	...	0.20	...	0.20	...	0.10	...	...	0	0	...
2[b]	5 T	0.83	...	0.6	...	0.50	...	...	0.33	...	0.1[d]	...
	C	0.60	...	0.45	...	0.35	...	...	0.20	...	0.1[d]	...
	2 T	1.00	...	0.73	...	0.66	...	...	0.40	...	0.2[d]	...
	C	0.65	...	0.65	...	0.45	...	...	0.15	...	0.05[d]	...
3[b]	1 T	...	1.1	...	0.66	...	...	0.28	...	...	0.13[d]	...
	C	...	0.6	...	0.5	...	...	0.20	...	...	0.10[d]	...
	0.5 T	...	1.0	...	0.75	...	...	0.55	...	...	0.15[d]	...
4[b]	5 T	0.90	...	0.60	...	0.40	...	...	0.26	...	0.06[d]	...
	C	0.60	...	0.45	...	0.35	...	...	0.20	...	0.10[d]	...
	2 T	0.60	...	0.46	...	0.26	...	...	0.13	...	0.06[d]	...
	C	0.65	...	0.65	...	0.45	...	...	0.15	...	0.05[d]	...
5[b]	5 T	...	1.50	...	1.10	...	0.73	...	...	0.26	...	0.20[e]
	C	...	1.70	...	1.10	...	0.80	...	...	0.43	...	0.23[e]
	2 T	...	0.60	...	0.36	...	0.30	...	0.1	...	...	0
	C	...	0.63	...	0.40	...	0.23	...	0.1	...	...	0
6[b]	1 T	...	0.40	...	0.20	...	0.15	...	...	0.10	...	0
	C	...	0.30	...	0.20	...	0.15	...	...	0.10	...	0
	0.5 T	...	0.20	...	0.20	...	0.10	...	...	0	...	0
	C	...	0.20	...	0.20	...	0.10	...	...	0.10	...	0

[a] Numbers refer to compounds listed in Table I.
[b] Numbers refer to compounds listed in Table IV.
[c] Complete healing on fourteenth day. [d] Complete healing on thirteenth day.
[e] Complete healing on sixteenth day. T Test. C Control.

Formulations.—In order to determine the synergistic or antagonistic behavior of the selected antifungal compounds, different formulations were prepared, containing a few commonly used antifungal agents. The two most effective antibacterial compounds as shown by these studies were incorporated in some of the mixtures.

The serum agar-cup-plate method described earlier was used with *T. gypseum* as the test organism. For every formulation two mixtures were prepared; one of these (*a*) contained that concentration of each substance which was able to produce a zone diameter of 16 mm. as determined previously (see under influence of organic matter) and the other (*b*) one-half of those concentrations. Hence if a zone diameter of more than 16 mm.was shown by mixture (*a*), and at least 16 mm. by mixture (*b*), the test substances in those mixtures were considered to be synergistic in their action. Where antibacterial compounds were added in the mixtures, the concentrations used were twenty times their minimum inhibition concentrations against *M. pyogenes* var. *aureus*, determined previously (1).

The results recorded in Table VII indicate that 6-bromo- and 1:6-dinitronaphthols appear to be synergistic with some of the known antifungal agents studied. Similar behavior is shown by 6-hexyl-β-naphthol when combined with 8-hydroxyquinoline. Further detailed studies on these and other combinations are in progress.

TABLE VII.—FUNGISTATIC ACTIVITY OF FORMULATIONS CONTAINING β-NAPHTHOL DERIVATIVES AND KNOWN ANTIFUNGAL AGENTS

Formulation	Concn., Respectively, in Final Mixture, %	Av. Zone Diameter, mm. I[a]	II[b]
UDA & SA	2.5, 0.1	14.0	13.0
UDA & 8Q	2.5, 0.05	17.0	15.5
SA & 8Q	0.10, 0.05	19.0	15.0
UDA & 6-Br	2.5, 0.025	15.0	14.5
UDA & Dinitro	2.5, 0.010	15.5	15.0
UDA & 6-Br & Dinitro	2.5, 0.025, 0.010	14.0	14.0
SA & 6-Br	0.1, 0.025	17.0	14.0
SA & Dinitro	0.1, 0.01	17.0	14.0
SA & DiBr	0.1, 0.001	15.0	13.5
8Q & 6-Br	0.05, 0.025	18.0	16.0
8Q & Dinitro	0.05, 0.01	18.0	15.0
8Q & Hexyl	0.05, 0.005	19.0	14.5

[a] With concentrations given in column 2.
[b] With half the concentrations given in column 2.
UDA = Undecylenic acid; 6-Br = 6-Bromo-β-naphthol; Dinitro = 1:6-Dinitro-β-naphthol; DiBr = 1:6-Dibromo-2-hydroxy-3-naphthanilide; Hexyl = 6-*n*-Hexyl-β-naphthol; SA = Salicylanilide; and 8Q = 8-Hydroxyquinoline.

SUMMARY

Some derivatives of β-naphthol selected from a previously reported preliminary antibacterial and antifungal screening have been evaluated for their potential value as anti-infective agents. Methods of sterilization, penetration power, influence of organic matter, and fungicidal activity were some of the properties examined. The more effective antibacterial compounds, such as 6-*n*-hexyl-β-naphthol, 1:4′-, and 1:6-dibromo-2-hydroxy-3-naphthanilides, and the antifungal compounds, 1-bromo-, 6-bromo-, and 1:6-dinitro-β-naphthols, have also been studied for topical irritation and interference in the process of epithelization. Further, the anti-inflammatory activity and the acute toxicity of the bacteriostatic compounds have been determined. Finally, some formulations containing the naphthol derivatives and certain known antifungal agents were examined for any potentiation of fungistatic action.

1-Bromo-, 6-bromo-, 1:6-dinitro-, and 6-*n*-hexyl-β-naphthols and 1:6-dibromo-2-hydroxy-3-naphthanilide were found to possess good penetration power in the form of ointments and were nonirritant to the intact skin of rabbits. They did not seem to interfere in the process of healing of wounds nor were their activities seriously affected by organic matter. A synergistic action of some of these compounds was observed in formulations containing certain useful antifungal agents. The LD_{50} by intraperitoneal administration of 6-hexyl-β-naphthol was of the order of 127 mg./Kg. It appears from these results that the compounds may prove to be useful anti-infective agents and hence merit clinical study.

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The Excretion and Tissue Distribution of C^{14}-Carbonyl Salicylamide in the Rat*

By WILLIAM F. BOUSQUET and JOHN E. CHRISTIAN

This investigation was undertaken to determine the excretion and tissue distribution of C^{14}-carbonyl labeled salicylamide and its metabolites in the rat. Twenty-four hours following the administration of 250 mg./Kg. of salicylamide containing 2.35 μc. of C^{14}, 96.9 per cent of the administered radioactivity was found in the urine. Thirty minutes following the oral administration of 250 mg./Kg. of the compound containing 4.35 μc. of C^{14} the greatest quantity of the drug and its metabolites was found in the kidney. The brain-plasma ratio expressed on a dry weight basis was found to be 1.72.

THE ANALGETIC VALUE of salicylamide is well recognized (1, 2) and has been reported by some workers to be greater than that of aspirin (3). It appears that salicylamide differs greatly from other members of the salicylate group of drugs in its metabolism and mode of action. The findings of Brodie and Szekely (4) would indicate that the compound is not converted to salicylate in the animal body.

Salicylamide seems to be free of the untoward effects often noted with aspirin therapy (5, 6). The compound appears to be well absorbed and no gastric ulceration has been noted upon prolonged administration. Changes in red and white cell counts or in prothrombin time have not been reported to occur with salicylamide therapy (6).

The overall excretory pattern of salicylamide was studied by administering the drug labeled with C^{14} to rats, collecting the urine and feces separately over a period of twenty-four hours, and analyzing the excretion products for their radioactive content.

To determine the tissue distribution of the drug and/or its metabolites various tissues and organs were taken for assay thirty minutes following the oral administration of the compound labeled with C^{14}. These organs and tissues were then brought into a suitable form for analysis and their radioactive content determined.

EXPERIMENTAL

Synthesis of C^{14}-Carbonyl Salicylamide.—The labeled compound used in this study was prepared using C^{14}-carboxyl salicylic acid previously synthesized in this laboratory by Borst and Christian (7). The procedure reported by Mandel, *et al.* (8), was modified for use in this work. The synthesis was carried out by refluxing 0.5648 Gm. of C^{14}-carboxyl salicylic acid representing 1.51×10^9 d. p. m. of radioactivity with 8.0 ml. of absolute methanol for twenty-four hours in the presence of one drop of concentrated sulfuric acid. When the refluxing period was complete 10 ml. of cooled 28% ammonia solution was added to the reaction mixture and the flask shaken mechanically for eighteen hours.

The reaction mixture was then cooled in an ice bath and the salicylamide and unreacted salicylic acid precipitated by addition of cold 20% hydrochloric acid added dropwise. After refrigeration for two days during which time cold 20% hydrochloric acid was added intermittently to insure completeness of precipitation, the mixture was suction filtered and the salicylamide and unreacted salicylic acid collected. The filtrate and washings were then concentrated, cooled, and allowed to crystallize. This procedure was repeated three times. All solid material obtained in the crystallizations was combined and this material was then treated with a saturated solution of sodium carbonate to remove any free salicylic acid present. Total yield of C^{14}-carbonyl salicylamide was 0.4039 Gm. or 72% of theory before recrystallization. Recrystallization from water yielded 0.2960 Gm. of material having a melting point of 140° (M.M.P.-K) which is in agreement with the literature value for salicylamide (9). Total yield of purified product was 52% of theory.

Demonstration of Chemical and Radiochemical Purity.—Purity of the labeled compound was ascertained by subjecting it to chromatographic and autoradiographic analysis. An acetone solution of the synthesized C^{14}-carbonyl salicylamide was prepared in a concentration of 30 gamma per 5 lambda. A 5 lambda sample of this solution was chromatographed on Whatman No. 1 paper using the descending method of paper partition chromatography. The solvent system employed was benzene-glacial acetic acid-water (40 : 40 : 20). Reference samples of unlabeled salicylic acid and salicylamide were run as controls on the same paper. The R_f value of the synthesized C^{14}-carbonyl salicylamide was determined to be 1.0, indicating that the labeled compound was identical with the salicylamide reference compound. No salicylic acid could be demonstrated to be present in the synthesized C^{14}-carbonyl salicylamide upon color development of the chromatogram with a 1% solution of ferric chloride.

To demonstrate the absence of radiochemical impurities in the synthesized compound an autoradiogram of the chromatogram was prepared. Eastman

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Kodak "No-Screen" X-ray film was used in preparing the autoradiogram and the exposure time was calculated on the basis of the known activity present on the chromatogram. Upon film development there was present but one spot showing that there were no radiochemical impurities present in the synthesized product.

Activity Determination.—Samples of the synthesized C^{14}-carbonyl salicylamide were dissolved in acetone for determination of their specific activities. The activity[1] of aliquots of the synthesized C^{14}-carbonyl salicylamide showed that the product had a specific activity of 1.18 μc./mg.

Studies of Salicylamide Excretion.—To each of five white female rats of the Purdue-Wistar strain (average weight of each was 202 Gm.) was administered 250 mg./Kg. of salicylamide containing 2.35 μc. of C^{14}. Sufficient labeled material to give the desired activity was ground in a glass mortar with unlabeled salicylamide, wetted with a few drops of Aerosol OT,[2] and suspended with the aid of a 0.5% solution of Kelgin XL.[3] The suspension was administered to the animals by means of a stomach tube.

Upon administration of the drug each animal was placed in a metabolism chamber similar to that described by Roth, *et al.* (10), which is equipped to mechanically separate the urine and the feces. For sixteen hours prior to determining salicylamide excretion and during the entire twenty-four hour period of study the animals were allowed water freely but food was withheld. The animal was held in the chamber and the urine and feces collected over a twenty-four hour period.

The urine and feces samples from the five animals were pooled and the aliquot assayed taken as a representative sample of the excretory products of the five animals. The feces sample was homogenized with 10% nitric acid to provide uniform sampling. Samples of the excretion products were then deposited on aluminum counting planchets of 7.31 $cm.^2$ area, and the radioactivity of the samples determined with the counting set up as previously described.

Upon assay of the excretory samples it was found that 96.9% of the administered radioactivity was excreted in the urine and that 0.61% was found in the feces. Clearly, then, the kidney represents the organ of excretion of this drug.

Determination of the Tissue Distribution of C^{14}-Carbonyl Salicylamide and/or Its Metabolites.—In order to determine the distribution in the animal body of salicylamide and/or its metabolites a dose of salicylamide corresponding to 250 mg./Kg. and containing 4.35 μc. of C^{14} was administered to five white female rats of the Purdue-Wistar strain, the average weight of which was 211 Gm. Prior to administration of the drug the animals were fasted for twenty-two hours but were allowed water freely.

The drug was prepared and administered as previously described. Thirty minutes later the animals were sacrificed by decapitation, the blood being collected and pooled in a graduated centrifuge tube containing ammonium oxalate solution. The blood was centrifuged at 3,000 r. p. m. for thirty minutes and the clear plasma removed by means of a pipet. To the red blood cells remaining was added an equal volume of distilled water in which the cells were allowed to hemolyze for ten hours.

The heart, lung, spleen, liver, adrenals, brain, and kidneys of the animals were removed, pooled, and homogenates prepared in 10% nitric acid with the aid of a tissue grinder. The homogenates were prepared such that a 0.250-ml. sample would contain approximately 10 mg. of dry weight of tissue. Tissue and blood samples were prepared in duplicate for determination of their specific activities. All samples were corrected for self absorption, background, and efficiency of the counting set up. Counting was carried out sufficiently long to provide results within a maximum error of 5%. Table I presents the data obtained. The figures given are a numerical average of duplicate determinations.

TABLE I.—TISSUE DISTRIBUTION OF SALICYLAMIDE AND ITS METABOLITES THIRTY MINUTES AFTER THE ORAL ADMINISTRATION OF C^{14}-CARBONYL SALICYLAMIDE TO THE RAT

Sample	Activity, d. p. m./mg., Wet Wt.	Activity, d. p. m./mg., Dry Wt.	Tissue-Plasma Ratio, Wet Wt. Basis	Tissue-plasma Ratio, Dry Wt. Basis
Plasma	32	532	1.00	1.00
R. B. C.	...	195	...	0.36
Kidney	296	1,435	9.20	2.70
Brain	218	917	6.50	1.72
Heart	168	672	5.25	1.36
Lung	53	271	1.65	0.51
Spleen	118	503	3.70	0.94
Liver	73	306	2.25	0.57
Adrenal	29	141	0.90	0.26

DISCUSSION

From the results obtained in the determination of the excretion of salicylamide and its metabolites it is clear that urinary excretion plays the major role in this species. The low level of activity found in the feces is undoubtedly due to contamination by urine and does not indicate excretion of the drug by this route.

It appears significant that saliclyamide and/or its metabolites are found in brain tissue in relatively higher concentration than in other tissues with the exception of the kidney. This accumulation warrants further studies as a possible explanation of the higher level of analgetic activity of salicylamide as opposed to others of the salicylate group of drugs reported by several authors (1-3).

SUMMARY

The synthesis of C^{14}-carbonyl salicylamide from C^{14}-carboxyl salicylic acid by a modification of the procedure of Mandel, *et al.* (8), has been

[1] All radioactivity determinations in this work were made utilizing a Nuclear Instrument and Chemical Corp. end window tube of 1.4 mg./$cm.^2$ thickness in combination with a Radiation Instrument Development Laboratories Model 512 decade scaling unit. The tube was shielded with lead, the background ranging from 12–18 c. p. m. The efficiency of this counting set up for C^{14} was determined as being 2.55% utilizing a National Bureau of Standards C^{14} Sodium Carbonate Standard.

[2] American Cyanamid Co.

[3] Sodium Alginate, Low Viscosity, Kelco Co., N. Y.

described. Identification and radiochemical purity have been established by a combination of chromatography and autoradiography.

A study of salicylamide excretion during a twenty-four hour period following the oral administration of 250 mg./Kg. of salicylamide containing 2.35 μc. of C^{14} in the presence of a nonionic surfactant revealed that 96.9 per cent of the administered dose was excreted in the urine and that 0.61 per cent was found in the feces.

The tissue distribution of salicylamide and its metabolites in the rat thirty minutes after the oral administration of 250 mg./Kg. of salicylamide containing 4.35 μc. of C^{14} in each dose and in the presence of a nonionic surfactant has been determined for the lung, heart, kidney, brain, liver, spleen, adrenals, plasma, and the red blood cells. The greatest accumulation of the drug and/or its metabolites was found in the kidney. The relatively high concentration of the drug in the brain tissue is indicative of the ability of this drug to penetrate the blood-brain barrier to a greater extent than has been reported for the salicylates.

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A Histological Study of Some Mucilaginous Seeds*

By MAHMOUD D. SAYED† and JACK L. BEAL

A description of the morphology and histology of the seeds of Kentucky coffee-tree, thornless honey locust, and sweet basil is given. Mucilage is located in the endosperm of the first two seeds, and in the epidermis of the seed coat of the third.

THE ECONOMIC IMPORTANCE as well as the pharmaceutical and therapeutic importance of certain types of mucilaginous seeds is a well-established fact. This paper concerns a histological study of mucilaginous seeds of three different plants. They are *Gymnocladus dioica* (L.) Koch., kentucky coffee-tree; *Gleditsia triacanthos* L. var. *inermis*, thornless honey locust; and *Ocimum basilicum* L., sweet basil. The seeds of these plants are of potential commercial importance since seeds of the first two possess a mucilage similar to guar mucilage while the third is more of the order of plantago seeds. Current research is being carried out at this institution on the physical properties and the chemistry of the mucilage of these seeds. It is anticipated that the results will be published at a later date.

KENTUCKY COFFEE-TREE

Gymnocladus dioica (L.) Koch. of the *Leguminosae* family, commonly known as the kentucky coffee-tree, is a tree 20 to 25 meters in height. It has bipinnate leaves; the leaflets being ovate or oval and range from 2.0 to 7.0 cm. long. The flowers are terminal racemes and are described as polygamous, regular. The fruits are pods, being flat and oblong with a very hard texture (1).

Morphology of the Seed.—Kentucky coffee beans, Fig. 1, are very hard button-shaped seeds varying from 1.5 to 2.5 cm. in length, and 0.3 to 0.5 cm. in thickness. The seed coat is smooth and dark brown in color. On the circumference, there is an oval depression marking the position of micropyle and hilum. The seed is albuminous. The endosperm is mucilaginous and swells enormously when brought in contact with water.

A longitudinal section parallel to the flat surface, Fig. 1,[1] shows a large circular cotyledon, filling most of the concavity of the seed, and a small conical radicle directed toward the micropyle. The embryo is surrounded by a narrow translucent mucilaginous endosperm.

Histology.—The seed coat consists of an epidermis, a hypodermis, an intermediate (nutrient) layer, and an innermost collapsed layer.

The epidermis, Fig. 2, consists of palisade-like

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[1] The pictures in this paper were photostated by the Photography Department, The Ohio State University.

cells varying from 18.5 to 23.0μ in height and from 0.9 to 1.2μ in thickness. They have thick cellulosic walls and are covered with a thick cuticle. In surface view, Fig. 3, the epidermal cells appear polygonal in outline with circular lumina. The epidermal tissue was marked by a horizontal line which appeared approximately 6.5μ from the apex. This was of some concern for it gave the appearance of the tissue being made up of two rows of cells; a long row and a short row joined end to end, rather than one row. It was proved to be one row by measuring the length of the cells while intact in the tissue and then measuring them after being separated from the tissue by maceration. Their length within the tissue and after separation was found to be the same indicating that the epidermal tissue consists of one row of cells having the appearance of two rows. In the study of the isolated cells, the appearance of this line was not so distinct as in the transverse section. It is probably due to a difference in the refractive index of the cell wall in the particular area. This phenomenon is to be studied further at this institution.

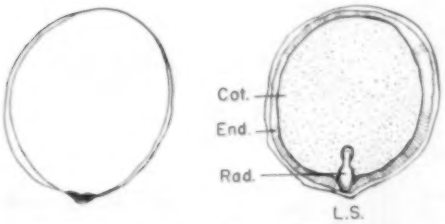

Fig. 1.—Kentucky coffee. ×3. Longitudinal section (L. S.); cotyledon (Cot.); endosperm (End.); radicle (Rad.). ×2.

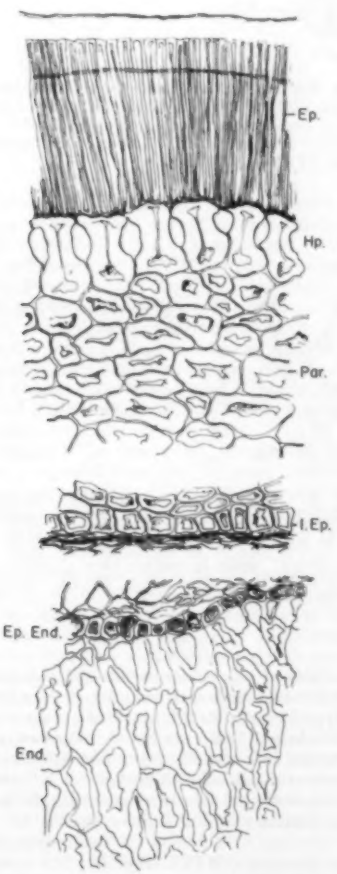

Fig. 2.—Transverse section of Kentucky coffee seed. Epidermis (Ep.); hypodermis (Hp.); intermediate parenchyma (Par.); inner epidermis of seed coat (I. Ep.); epidermis of endosperm (Ep. End.); endosperm (End.). ×145.

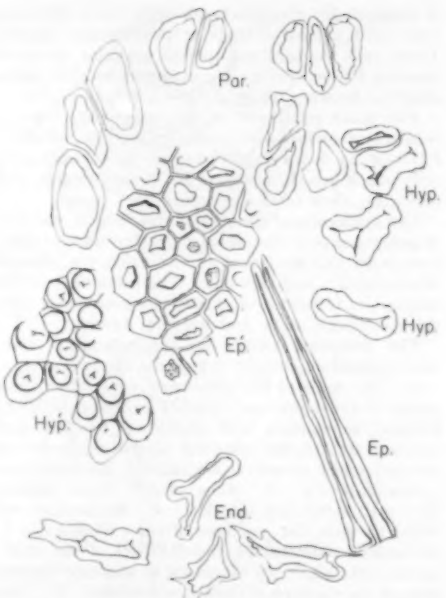

Fig. 3.—Isolated elements of Kentucky coffee seed. Epidermis of seed coat in lateral view (Ep.); same in surface view (Ep'.); intermediate parenchyma (Par.); endosperm cells (End.); hypodermis in lateral view (Hyp.); same in surface view (Hyp'.). ×148.

The hypodermis, Fig. 2, consists of one layer of beaker cells having the shape of short-necked bottles, with cellulosic-thickened walls and narrow lumina which widen at both extremities of the cells assuming irregular triangular shapes. Between these cells there are wide oval intercellular spaces. These cells vary from 4.9 to 5.6μ in height and 1.7 to 2.3μ in breadth (at the broadest part), and 0.6 to 0.9μ at the narrowest part (neck). In surface view, Fig. 3, they appear circular or oval in outline with stellate lumina.

The intermediate parenchymatous tissue (nutrient layer) consists of 9 to 12 layers of isodiametric

or tangentially elongated cells with thick cellulosic walls and narrow triangular intercellular spaces. These cells contain amorphous granular contents reacting for protein by staining yellow with picric acid and brown with iodine test solution.

The inner epidermis of the seed coat, Fig. 2, consists of one row of rectangular cells. This is followed by a layer of collapsed parenchymatous cells which are colored blue by iodine-sulfuric acid indicating their cellulosic nature.

The epidermis of the endosperm, Fig. 2, consists of polygonal cells containing numerous minute aleurone grains that are free of any inclusion, i. e., devoid of crystalloids and globoids. The remainder of the endosperm is formed of axially elongated cells with thick cellulosic walls and irregular lumina.

The following additional microchemical tests were applied on a series of sections of the seed:

1. The walls of the endosperm cells, other than those of the epidermis, swelled enormously when brought in contact with water. 2. Iodine test solution stained the contents of the epidermis of the endosperm as well as the cells of the cotyledons yellowish-brown. 3. None of the tissue stained blue with iodine test solution. 4. Ruthenium red failed to stain the mucilage of the endosperm. 5. Methylene blue stained the contents of the endosperm cells blue. 6. Fuchsin in absolute alcohol stained the contents of the endosperm red. 7. The contents of the embryo stained red with Sudan III test solution. 8. Chlorzinciodine stained the cell walls of the endosperm and embryo violet. The contents of the endosperm and embryo cells stained yellow to yellowish-brown. 9. The cell walls did not give a positive lignin test.

From the above tests one may conclude the following:

1. The cell walls were nonlignified. 2. The epidermis of the endosperm contains aleurone grains while the remainder of the cells of the endosperm contain mucilage. 3. The cells of the embryo contain fixed oils and aleurone grains. 4. Starch is absent in the cells of all the tissues.

THORNLESS HONEY LOCUST

Gleditsia triacanthos L. var. *inermis* of the *Leguminosae* family is commonly known as the thornless honey locust. The plant is a tree with bipinnate leaves, leaflets often crenulate. The flowers are small and are in axillary or lateral racemes or panicles. The fruit is a pod, linear, 2.0 to 3.0 cm. in length, curved, and twisted (2).

Morphology of the Seeds.—The seeds, Fig. 4, are oval varying from 0.5 to 1.0 cm. in length, 4.0 to 7.5 mm. in breadth, and 2.0 to 5.0 mm. in thickness. The seed coat is smooth and tan to brown in color. At one end of the seed there is a small oval depression marking the position of the embryo. The seed is albuminous. The endosperm is mucilaginous and swells with water and aqueous reagents. A longitudinal section of the seed, Fig. 4, shows a large flat cotyledon and a small radicle surrounded by a mucilaginous endosperm.

Histology.—The seeds of the thornless honey locust show the same arrangement and type of tissues as the seeds of the Kentucky coffee-tree. The following are the important diagnostic features of the powder (Fig. 5):

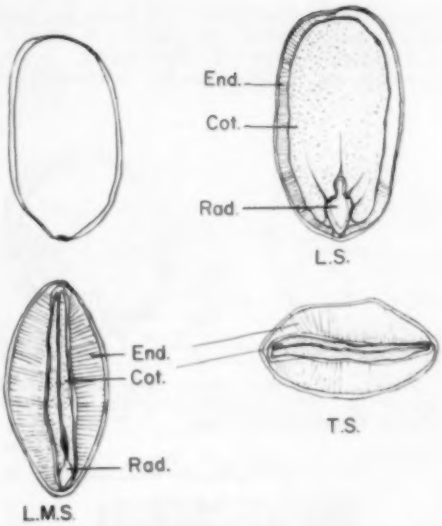

Fig. 4.– Honey locust seed. Longitudinal section parallel to the flat surface (L. S.); cotyledon (Cot.); endosperm (End.); radicle (Rad.); longitudinal median section (L. M. S.); transverse cut surface (T. S.). ×4.6.

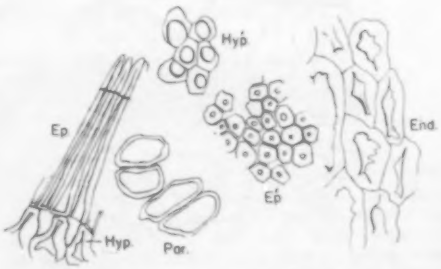

Fig. 5.—Isolated elements of honey locust seed. Epidermis of the seed coat in lateral view (Ep.); same in surface view (Ep.); hypodermis in lateral view (Hyp.); same in surface view (Hyp.); parenchyma of intermediate layer (Par.); endosperm (End.). ×110.

1. Cells of the epidermis are palisade-like and are 13.2 to 16.5μ in length and 0.6 to 0.9μ in breadth. 2. Hypodermal cells are basket shaped with cellulosic thickened radial walls. 3. Parenchymatous cells of the intermediate layer had cellulosic walls and were tangentially elongated. 4. Endosperm cells consisting of axially elongated cells with irregular lumina and mucilaginous contents.

SWEET BASIL

Ocimum basilicum L. of the *Labiateae* family, commonly known as sweet basil, is an annual plant much branched 30 to 60 cm. in height. The leaves are ovate, petiolate, 2.5 to 5.0 cm. in length, with

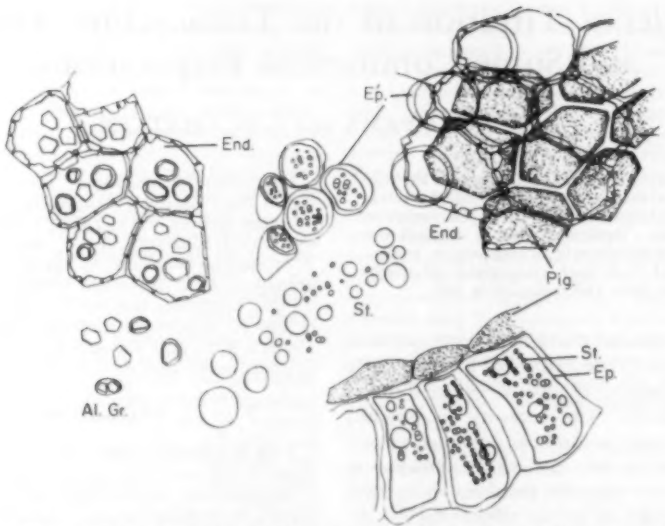

Fig. 7.—Isolated elements of sweet basil seed. Epidermis of seed coat in lateral view (Ep.); same in surface view (Ep.); starch grains (St.); pigment layer (Pig.); endosperm (End.); aleurone grains (Al. Gr.). ×110.

an entire or toothed margin. The flowers are white, or more or less tinged purple, in moderately dense racemes. *Basilicum* in Greek means kingly because of its healing property. It is native to tropical Asia and the Pacific islands (2).

Morphology of the Seeds.—The seeds are hard in texture, ovoid in shape, varying from 2.0 to 3.0 mm. in length and from 0.5 to 1.0 mm. in breadth. The seed coat is dark brown in color showing a minutely pitted surface when viewed with a magnifying lens. The seed is albuminous with an oily endosperm surrounding the minute embryo.

Histology.—The seed coat consists of an epidermis, a pigment layer, and an inner layer of large parenchymatous cells. The epidermis, Fig. 6, consists of large axially elongated cells varying from 13 to 19.5μ in height and 6.6 to 11.5μ in breadth. Their walls are cellulosic in nature. The radial walls are more thickened than the outer and inner tangential walls. These cells swell enormously when brought in contact with water and their walls stain blue to purplish blue with methylene blue indicating that they are impregnated with mucilage. The lumina of the cells are wide and contain numerous minute simple and a few 2- to 4-compound starch grains. In surface view, Fig. 7, these cells appear rounded with circular lumina. Underneath the epidermis there is one and occasionally two rows of tabular cells containing a dark brown content which is responsible for the color of the seed coat. In surface view, Fig. 7, they appear polygonal in outline usually adhering to the cells of the epidermis and endosperm. The innermost layer of the seed coat consists of one row of large parenchymatous cells with thin cellulosic walls. The storage tissue in these seeds consists of the endosperm and the two cotyledons.

The endosperm, Fig. 6, consists of isodiametric

Fig. 6. Transverse section in sweet basil seed. Epidermis (Ep.); pigment layer (Pig.); parenchymatous layer (Par.); aleurone grains (Al.); endosperm (End.); ×148.

cells. They have thick-beaded walls with distinct simple pits. Their contents stain red with Sudan III and yellowish-brown with iodine indicating the presence of oil and protein respectively. The protein is present in the form of oval aleurone grains, containing one and occasionally two crystalloids but no globoids.

The embryo cells contain numerous aleurone grains and oil globules.

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Nonaqueous Titration of the Tetracycline Antibiotics and Some Commercial Preparations*

By F. YOKOYAMA and L. G. CHATTEN

The application of visual nonaqueous titrimetry has been made to the assay of the tetracycline antibiotics and their commercial preparations. Determination is carried out in nitromethane-formic acid-benzene system using mixed indicator comprised of methylene blue and quinaldine red.

THE WIDE USE and the therapeutic importance of the tetracycline antibiotics has resulted in an increased number of pharmaceutical forms of these compounds. The necessity of carefully maintained quality control has prompted numerous investigations into analytical techniques in an effort to cope with the problems which have arisen. In addition to the microbiological determination using the turbidimetric and cylinder-plate methods a considerable number of fluorometric and colorimetric procedures have been proposed (1–12). On the other hand, very little has been published on the application of nonaqueous titrimetry as a chemical means of analysis of these preparations. Sideri and Osol (13) as well as Ekeblad (14) have reported the determination of some of the tetracyclines in glacial acetic acid, but we have been unsuccessful in reproducing satisfactory results by their method. The microbiological technique is still the most widely used, although many experts agree that the recoveries may deviate as much as plus or minus ten per cent from the mean value.

The purpose of this study is to investigate the application of nonaqueous procedures which can be readily performed by visual titration for both the free bases and their hydrohalide salts. The work has been extended to include tetracycline, chlortetracycline, and oxytetracycline compounds.

EXPERIMENTAL

Apparatus.—Five-cubic centimeter buret, graduated in 0.01 cc. or 10-cc. buret, graduated in 0.05 cc.; electromagnetic stirrer; Fisher titrimeter, (Model No. 9-311A); silver-silver chloride electrode, (Beckman No. 1264); and glass electrode (Fisher No. 9-312-25).

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Reagents.—Nitromethane, practical grade, obtained from Eastman Kodak (redistilled, fraction collected between 100 and 101°); benzene, A. C. S.; formic acid, 85 to 90%, A.C.S.; perchloric acid, 0.05 *N* in dioxane, standardized against potassium acid phthalate, A. C. S.; mixed indicator comprised of 0.1 Gm. methylene blue and 0.2 Gm. quinaldine red in 100 cc. anhydrous methanol (methyl hydrate, absolute B. D. H.); and mercuric acetate, C. P., 6% in glacial acetic acid, A. C. S.

PROCEDURES

Pure Antibiotics.—Accurately weigh a sample of about 50 mg. into a 150-cc. beaker, dissolve in 50 cc. of nitromethane containing 1 cc. of formic acid by stirring electromagnetically. Add 5 cc. of benzene, 1 cc. of 6% mercuric acetate in glacial acetic acid where a hydrochloride salt is to be analyzed, and 0.1 cc. of the mixed indicator. Titrate with 0.05 *N* perchloric acid in dioxane to the end point indicated by the appearance of the green color. Each cc. of 0.05 *N* perchloric acid is equivalent to: 25.77 mg. chlortetracycline (Aureomycin®, Lederle) hydrochloride, 24.85 mg. oxytetracycline (Terramycin®, Pfizer) hydrochloride, 23.02 mg. oxytetracycline (Terramycin®, Pfizer) base, 24.05 mg. tetracycline (Achromycin®, Lederle) hydrochloride, and 22.22 mg. tetracycline (Achromycin®, Lederle) base.

Commercial Preparations.—*Capsules and Tablets.*—Weigh and powder 20 tablets or weigh the contents of 20 capsules. Place an accurately weighed sample equivalent to 50 mg. of the drug in a 150-cc. beaker. Add 25 cc. of nitromethane containing 1 cc. of formic acid and stir electromagnetically for thirty minutes. Filter through a Whatman No. 1 filter paper into a 150-cc. beaker. Wash the residue with an additional 25 cc. of nitromethane. Add 5 cc. of benzene, 1 cc. of 6% mercuric acetate in glacial acetic acid, and 0.1 cc. of the mixed indicator. Titrate to a green end point with 0.05 *N* perchloric acid in dioxane. Where the formulation includes magnesium stearate, titrate to a blue end point before the addition of mercuric acetate, then add mercuric acetate and continue titration to a green end point.

Ointments.—(*a*) Chlortetracycline (Aureomycin) hydrochloride and Tetracycline (Achromycin) hydrochloride ointments.

Accurately weigh a sample equivalent to 50 mg. of drug in a 150-cc. beaker, add 2 cc. of formic acid, 50 cc. of nitromethane, 40 cc. of benzene and warm on a hot water bath until the ointment is uniformly dispersed. Add an additional 10 cc. of benzene, 1 cc. of 6% mercuric acetate in glacial acetic acid, and 0.1 cc. of the mixed indicator. Titrate while hot to a green end point with 0.05 *N* perchloric acid in dioxane.

(*b*) Oxytetracycline (Terramycin) hydrochloride ointment with polymyxin B sulfate.

Accurately weigh a sample equivalent to 50 mg. of

drug in a 150-cc. beaker, add 50 cc. of benzene and warm on a hot water bath until the ointment is uniformly dispersed. Stir electromagnetically while cooling. Filter through a medium porosity sintered glass funnel and extract the residue with 50 cc. of nitromethane by filling the funnel. Omit suction, allowing the filtration to proceed under the influence of gravity. Add 5 cc. of benzene, 1 cc. of formic acid, 1 cc. of 6% mercuric acetate in glacial acetic acid, and 0.1 cc. of the mixed indicator and titrate to a green color with 0.05 *N* perchloric acid.

Oxytetracycline (Terramycin) Hydrochloride Intravenous Injection.—Completely wash out the content of a single ampul labeled to contain 250 mg. of active constituent with 50 cc. of nitromethane containing 5 cc. of formic acid and stir electromagnetically for thirty minutes. Filter through a Whatman No. 1 filter paper into a 100-cc. volumetric flask. The volume of the solvents will be proportional according to the amounts of active ingredient present. For example, when a sample contains 500 mg. of antibiotic, the amount of formic acid and nitromethane will be doubled. Wash the residue, then make up to volume with the nitromethane. To an aliquot equivalent to 50 mg. of drug add enough nitromethane to total 50 cc., 5 cc. of benzene, 1 cc. of 6% mercuric acetate in glacial acetic acid, and 0.1 cc. of the mixed indicator. Titrate to a green end point with 0.05 *N* perchloric acid in dioxane.

Chlortetracycline (Aureomycin) Hydrochloride Suppositories.—Place as many suppositories as required to yield a solution to contain 200 to 250 mg. of antibiotic in a 150-cc. beaker. Add 50 cc. of benzene and warm on a hot water bath until the suppositories are uniformly dispersed. Stir the mixture electromagnetically and allow to cool. Filter the cooled solution through a medium porosity sintered glass funnel, washing the residue with an additional 50 cc. of benzene. Place the funnel filled with nitromethane and 2 cc. of formic acid in a beaker, then allow the mixture to filter through under the influence of gravity. Repeat with additional nitromethane and the remainder of the formic acid, the total volume having been calculated such that 1 cc. is present for each 50 mg. of drug. Wash the funnel with nitromethane, transfer to a 200-cc. volumetric flask and make up to volume with nitromethane. To an aliquot equivalent to 50 mg. of active constituent add sufficient nitromethane to total 50 cc., 5 cc. of benzene, 1 cc. of 6% mercuric acetate in glacial acetic acid, and 0.1 cc. of the mixed indicator. Titrate to a blue-green end point with 0.05 *N* of perchloric acid in dioxane.

DISCUSSION

Tetracycline antibiotics, by virtue of the secondary amine group, can be determined as bases by direct titration with a strong acid such as perchloric. Both the free base and the hydrohalide salt can be assayed, the latter being dependent upon the addition of mercuric acetate solution as reported by Pifer and Wollish (15).

Of the numerous solvents or solvent combinations investigated, the nitromethane-formic acid pair employed by Streuli (16), in the titration of basic copolymers of acrylonitrile, was found to be the most satisfactory, particularly when combined with benzene. The addition of 2% by volume of formic acid to nitromethane appeared to solubilize the compounds completely, and thus eliminated the use of heat which was sometimes found necessary with glacial acetic acid. With some of the antibiotics, addition of formic acid was not required to effect complete solution but in order to maintain uniformity of the solvent system, however, it was used in all instances. With the use of 85–90% formic acid, the maximum amount of water introduced was calculated to be approximately 0.55% by volume, which according to Pifer, *et al.* (15), and Fritz, *et al.* (17), should cause no appreciable decrease in the sharpness of the potentiometric end point. Pifer, *et al.* (18), reported that the addition of a solvent with a low dielectric constant like benzene, chloroform, or dioxane can in many cases contribute to an increase of the potentiometric break at the end point. Of those investigated, benzene proved to be the most satisfactory.

With the proposed solvent mixture, the electrode combination of silver-silver chloride and glass was found to give a better potentiometric break at the end point in comparison to that obtained with calomel and glass. Another factor in favor of the former pair is that it is not necessary to periodically replace the calomel electrode salt bridge which is known to be readily contaminated by the mercuric acetate.

The main difficulty in finding a suitable indicator was caused by the inherent yellow color of the antibiotic, which created a considerable decrease in the sharpness of the color change at the end point. A few of the indicators which appeared to change at the potentiometric end point were: α-naptholbenzein, quinaldine red, bromothymol blue, and tropaeolin 00. However, with all of these, the end-point change was masked by the color of the antibiotic. For example, the usual red of tropaeolin 00 at the equivalence point was altered to red-orange, and, to overcome this difficulty, mixed indicators were tried. Methylene blue, which remained unchanged throughout the titration was chosen to provide the background color. Tropaeolin 00, when used alone changed from yellow to red-orange, but with the blue background provided by the methylene blue, the corresponding change was from green to purple which appeared to be easier to see visually. However, one could not determine the blank quite as accurately because of the less definite change, namely from blue-purple to red-purple. The mixed indicator which exhibited the most satisfactory result was the combination of methylene blue with quinaldine red where the color transition was from purple to blue, blue-green, or green depending upon the preparation. The determination of the blank was also improved with the change from purple to a definite blue. The proportion of each component in the screened indicator was found to play an important part, the most satisfactory one being outlined under the heading of reagent. Methanol was the solvent of choice, because the color did not fade back as it did to some extent when the indicators were dissolved in glacial acetic acid. The reproducibility of the results show that this solvent system and indicator combination is superior to that of Sideri and Osol (13) which enabled one to carry out only potentiometric determination with some of the compounds.

With chlortetracycline hydrochloride and tetra-

cycline base, the color of the indicator which coincided with the potentiometric end point was blue as shown in Figs. 1 and 2. However, it was noted that an increment of 0.01 cc. of the titrant altered the color from blue to green. For visual determination it was believed that a change from purple to green was more readily detected than the change from purple to blue. Hence, the green end point was selected with the full realization that it was 0.01 cc. of titrant beyond the true end point. It was thought that the error introduced was insignificant and was compensated for by the reproducibility of the results. However, with the tetracycline hydrochloride and oxytetracycline hydrochloride the color of the indicator at the potentiometric end point was green (see Figs. 3 and 4). Therefore, with all the pure antibiotics, titration was carried beyond the blue to the green color. Potential changes at the equivalence point were found to be approximately 20 to 30 millivolts for each 0.02 cc. of 0.05 *N* perchloric acid. To test the precision of this method 5 to 10 consecutive determinations were carried out and the standard deviations calculated. In addition, a comparison was made between the results obtained by the microbiological and spectrophotometric procedures. From the results recorded in Table I, one can clearly note the close agreements in the recoveries determined by these three methods, especially between the microbiological and the nonaqueous procedures. It is also apparent that the standard deviation is smallest with the nonaqueous method.

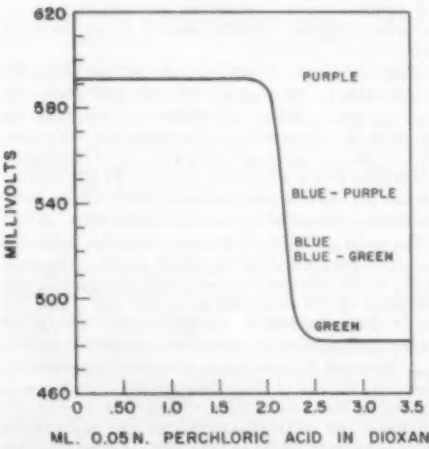

Fig. 1.—Titration of chlortetracycline hydrochloride in nitromethane-formic acid-benzene system.

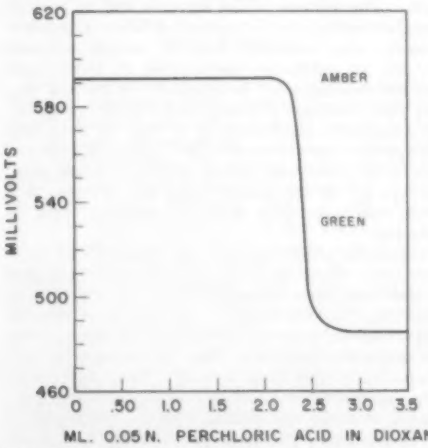

Fig. 3.—Titration of tetracycline hydrochloride in nitromethane-formic acid-benzene system.

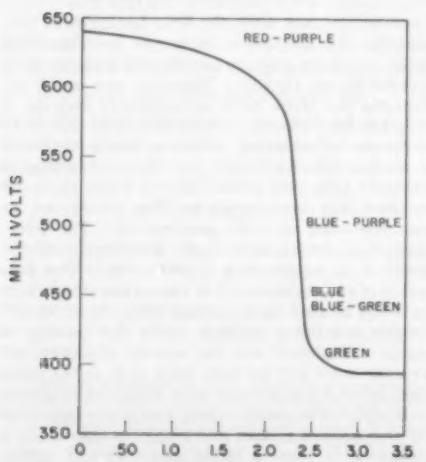

Fig. 2.—Titration of tetracycline base in nitromethane-formic acid-benzene system.

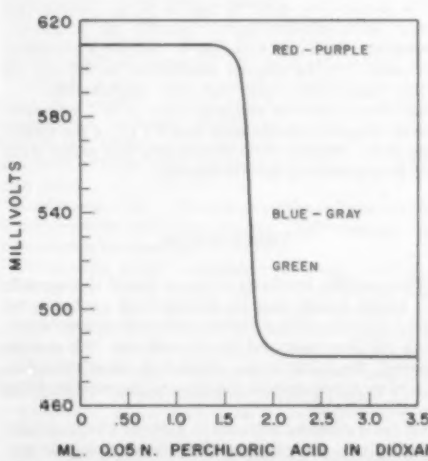

Fig. 4.—Titration of oxytetracycline hydrochloride in nitromethane-formic acid-benzene system.

TABLE I.—COMPARISON OF RECOVERIES OF THE PURE TETRACYCLINE ANTIBIOTICS

Compound	Nonaqueous Method	Microbiological Method	Spectrophotometric Method	
Chlortetracycline hydrochloride	99.46 ± 0.13 (9 det.)	991 μg./mg. or 99.1% ± 1.0 (7 det.)		
Tetracycline hydrochloride	99.88 ± 0.30 (10 det.)	1000 μg./mg. or 100% (1 det.)	96.3 ± 1.8 (3 det.)	Acid colorimetric method (1)
Tetracycline base	93.53 ± 0.46 (9 det.)	1036 μg./mg. 95.9% ± 2.3 (6 det.)	92.9 ± 1.9 (3 det.)	Same as with tetracycline hydrochloride
Oxytetracycline base	91.93 ± 0.62 Calc. as base $C_{22}H_{24}N_2O_9$ or 99.16 ± 0.67 Calc. as hydrochloride $C_{22}H_{24}N_2O_9$ HCl (6 det.)	929 μg./mg. or 100.2% ± 1.6 Calc. as hydrochloride $C_{22}H_{24}N_2O_9$ HCl (3 det.)	95.9 ± 0.0 (3 det.)	Ferric chloride colorimetric method (1)

When the commercial products were investigated, it was found necessary to modify the basic procedure in some instances due to interfering substances like the tablet excipients and ointment bases.

Since magnesium stearate, commonly employed as lubricant in tablets or as diluent in capsules, will react with the perchloric acid, the method required slight modification. This compound was extracted by the nitromethane-formic acid mixture resulting in apparent overestimation of the antibiotic. However, most of these preparations contained the antibiotic as the hydrochloride salt, and therefore, by titrating before the addition of mercuric acetate, the stearate was removed from further competition for the titrant. The volume of titrant consumed after the addition of mercuric acetate is a direct measure of the hydrochloride salt of the antibiotic. Thus, if any of the free base is present along with the hydrochloride salt, the former is not included in the final titration. This may account, in part, for the discrepancies between the microbiological or spectrophotometric methods and the proposed nonaqueous procedure (see Table II). To ascertain if the antibiotic was completely extracted during the proposed time of stirring, the procedure was repeated with the residue remaining after filtration. A blank titration showed that the antibiotic had been completely extracted. Unfortunately, the terramycin tablet could not be assayed by this method because the active constituent was present as the free base. It was impossible to determine the volume of the titrant consumed by the stearate because both the stearate and the terramycin base reacted with the perchloric acid without the addition of mercuric acetate. Therefore no results are reported for this preparation.

With the ointments it was found necessary to increase the volume of benzene in order to keep the ointment base in solution. This increase adversely affected the solubility of the antibiotic in the solvent combination and thus it necessitated the doubling of the amount of formic acid. Titration was carried out while hot because the mixture became turbid upon cooling. When the same procedure was re-

TABLE II.—COMPARISONS OF THE RECOVERIES OF THE TETRACYCLINE ANTIBIOTICS IN VARIOUS DOSAGE FORMS BY THE NONAQUEOUS, MICROBIOLOGICAL, AND SPECTROPHOTOMETRIC METHODS

Dosage Form	Antibiotic	Nonaqueous Method	Microbiological Method[a]	Spectrophotometric Method[b]
Capsule	Aureomycin hydrochloride	96.01 ± 0.41 (5 det.)	114 (1 det.)	108.3 ± 0.2 (3 det.)
	Achromycin hydrochloride	99.72 ± 0.66 (6 det.)	107 (1 det.)	103.2 ± 1.1 (3 det.)
	Terramycin hydrochloride	102.7 ± 0.75 (8 det.) Calc. as free base $C_{22}H_{24}N_2O_9$	103 (1 det.)	105.9 ± 1.6 (3 det.) Calc. as free base $C_{22}H_{24}N_2O_9$
Tablet	Achromycin hydrochloride	98.96 ± 0.29 (5 det.)	115 (1 det.)	103.2 ± 1.1 (3 det.)
Ointment	Aureomycin hydrochloride	115.5 ± 0.69 (5 det.)	114 (1 det.)	123.2 ± 0.70 (2 det.)
	Achromycin hydrochloride	118.0 ± 0.53 (5 det.)	107 (1 det.)	118.6 ± 1.6 (3 det.)
	Terramycin hydrochloride with polymyxin B sulfate	106.5 ± 0.24 (5 det.) Calc. as free base $C_{22}H_{24}N_2O_9$	105 (1 det.)	110.5 ± 1.4 Calc. as free base $C_{22}H_{24}N_2O_9$
Intravenous injection	Terramycin hydrochloride	112.4 ± 0.38 (5 det.)	93 (1 det.)	117.7 ± 0.5 (3 det.)
Suppository	Aureomycin hydrochloride	105.8 ± 0.32 (6 det.)	109.2 (1 det.)	111.5 ± 2.3 (6 det.)

[a] Microbiological assays were carried out by the same techniques as for pure antibiotics following the extraction procedures laid down in the reference.

[b] Spectrophotometric assays were carried out by the same methods as for pure antibiotics following the extraction procedures given in the text of Grove and Randall (1). Single exception to this was the intravenous Terramycin hydrochloride, which was analyzed by the ultraviolet method (1).

TABLE III.—RECOVERY OF THE PURE ANTIBIOTICS FROM KNOWN CONTROL MIXTURES

Compound	Method	Recovery, %
Chlortetracycline hydrochloride	Same as with ointment	99.63
		99.56
		99.97
Tetracycline hydrochloride	Same as with ointment	99.49
		99.51
		100.70
Chlortetracycline hydrochloride	Same as with suppository	99.19
		99.38
		100.0
Control		99.22
Chlortetracycline hydrochloride Suppository		99.42
		99.74

peated with the pure antibiotics, no alteration of recovery or behavior was noted during the titration. Excellent agreement between the basic procedure (Table I) and the control (Table III) appeared to uphold the validity of this method. Potential changes at the equivalence point were found to be approximately 20 to 30 millivolts for each 0.02 cc. of titrant. The titration curve for the Achromycin hydrochloride ointment is shown in Fig. 5.

The method was further modified for the terramycin ointment because of the presence of polymyxin B sulfate. The latter compound was believed to cause a slight overestimation. Since the polymyxin B sulfate is insoluble in organic solvents, it was possible to separate the two drugs. The ointment base was first removed by preliminary treatment with benzene, leaving a solid residue comprised of the two antibiotics, and the terramycin hydrochloride was then extracted by nitromethane. Complete extraction of the terramycin hydrochloride was substantiated by repeating the procedure on the white residue remaining in the funnel. To ascertain that the benzene extract of the base contained no terramycin hydrochloride the procedure was repeated with the base as described in the section on suppositories. Potential changes at the end point were about 20 millivolts for each 0.02 cc. of titrant. When the procedure which was employed with the aureomycin and achromycin ointments was applied to this preparation, the recovery tended to be about 0.5% higher than when assayed by the preceding method.

Since the antibiotic was insoluble in benzene, the suppository base was removed by preliminary treatment with this solvent. The antibiotic, which remained as the residue, was then taken up in solution by allowing the solvent mixture to be in contact with the compound. In order to completely extract the antibiotic from the pores of the sintered glass funnel, it was found necessary to prolong the contact of the drug with the solvent and consequently suction was not desirable. Filtration was permitted to proceed by gravity. To ensure that no antibiotic was extracted during the removal of the base, the benzene filtrate was evaporated and the entire procedure was repeated with the residue. Assay of the control suppository prepared according to the manufacturer's formulation and also of the pure compound by the similar method appeared to uphold the validity of the procedure. By comparing Tables I and III one can clearly note the excellent agreement in the recoveries of the pure antibiotic when assayed according to the basic procedure and the modified method for the suppository. The potential changes were approximately 20 to 25 millivolts for each 0.02 cc. of titrant. Titration curve for this preparation is shown in Fig. 6.

The Terramycin hydrochloride injection offered no difficulty. Potentiometric changes at the equivalence point were approximately 30 millivolts for each 0.02 cc. of titrant as shown in Fig. 7.

The results of these investigations of the commercial preparations are outlined in Table II along with

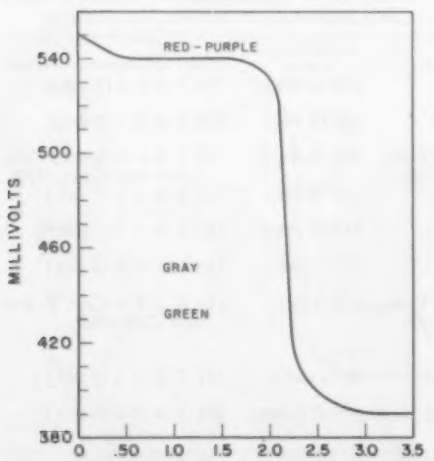

Fig. 5.—Titration of Achromycin hydrochloride ointment in nitromethane-formic acid-benzene system.

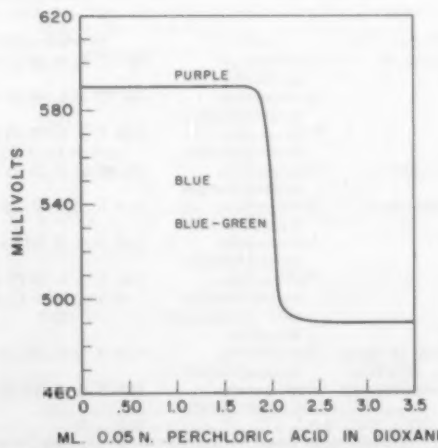

Fig. 6.—Titration of Aureomycin hydrochloride suppositories in nitromethane-formic acid-benzene system.

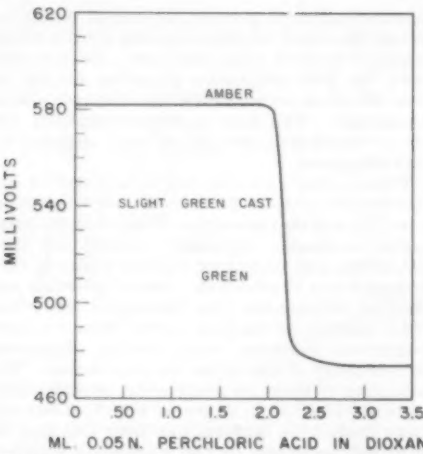

Fig. 7.—Titration of Terramycin hydrochloride intravenous injection in nitromethane-formic acid-benzene system.

the calculated standard deviations. Generally better agreement appears to occur between replicates with the nonaqueous procedures than with microbiological or spectrophotometric methods. The proposed technique seems to be preferable to that of Sideri and Osol (13) for both the pure compounds and some of their pharmaceutical preparations.

SUMMARY

1. A nonaqueous technique has been devised for the assay of the tetracycline antibiotics which is accurate and can be performed visually.

2. The procedure has been successfully applied to dosage forms including tablets, capsules, ointments, suppositories, and injectables.

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The Effects of Some Solid Buffering Agents in Aqueous Suspension on Prednisolone*

By THOMAS CHULSKI and ARLINGTON A. FORIST

The effects of aqueous suspensions of a number of solid buffers on prednisolone at 37.5° have been determined. The steroid was adsorbed by magnesium trisilicate according to the Freundlich relationship. Magnesium oxide suspensions produced a first-order degradation of the steroid side-chain to the etio acid. Aluminum hydroxide, calcium carbonate, and magnesium carbonate were without effect.

RECENTLY, several cortical steroid formulations containing buffering agents have appeared. Granulation procedures frequently used in the manufacture of such preparations involve exposure of the ingredients to moisture and elevated temperatures. In order to predict the effects of such treatment, the behavior of prednisolone ($\Delta^{1,4}$-pregnadien-11β,17α,21-triol-3,20-dione) has been determined in the presence of aqueous suspensions of a number of solid buffers at 37.5°.

* Received November 20, 1957, from The Upjohn Company, Kalamazoo, Mich.
The authors are indebted to L. M. Reineke and associates for the paper chromatographic analyses.

EXPERIMENTAL

Materials.—The buffers employed were as follows: (*a*) magnesium trisilicate Po., U. S. P., (*b*) magnesium oxide, heavy, U. S. P., (*c*) aluminum hydroxide, gel dried, U. S. P., (*d*) calcium carbonate, precipitated, U. S. P., and (*e*) magnesium carbonate, light, U. S. P. The prednisolone was 99.82% pure by solubility analysis. The blue tetrazolium reagent, 3,3′-dianisole-bis-4,4′-(3,5-diphenyl)-tetrazolium chloride was obtained from the Dajac Laboratories, Leominster, Mass.

Procedure.—In preliminary studies, 500 mg. of the solid buffer were mixed with 15 ml. of an aqueous solution of prednisolone (53–220 μg./ml.) in a 25-ml. volumetric flask. The flask was attached to a

Burrell wrist-action shaker mounted over a 37.5° constant temperature bath and the flask and its contents immersed in the bath and shaken for three hours. The solid was permitted to settle for approximately thirty minutes, still in the bath, and the supernatant solution was then withdrawn from the flask through a glass frit by means of vacuum. The resulting filtrate was analyzed for the conjugated carbonyl group of the steroid A-ring by ultraviolet spectrophotometry (243 mμ) and for the C_{17} α-ketol side-chain by the modification of the blue tetrazolium procedure of Mader and Buck (1) described below. The solid phase was leached with absolute ethanol and this solution likewise analyzed by the ultraviolet and blue tetrazolium procedures. Suspensions of the buffers without steroid were also carried through the above treatment in order to correct the analyses for any extraneous materials eluted from the solids. The pH of each suspension was determined at 37.5° by means of a Beckman Model G pH meter with glass-calomel electrodes.

In the determination of the adsorption isotherm for prednisolone on magnesium trisilicate, equilibration, and filtration were as above. However, only the ultraviolet analysis was applied to the filtrates as preliminary studies had shown adsorption of the steroid but no side-chain degradation.

In the kinetics studies of the degradation of the α-ketol side-chain in the presence of magnesium oxide, reaction mixtures were prepared as above. Shaking was stopped three minutes prior to the removal of each sample. A small portion of the supernatant solution was simultaneously withdrawn and filtered through a glass frit by means of vacuum. An aliquot of the filtrate (0.20–0.50 ml.) was immediately removed with a micropipet, the time noted, and the reaction quenched by the addition of the sample to 9.00 ml. of absolute ethanol. The resulting solution was analyzed by the modified blue tetrazolium procedure since preliminary studies had indicated degradation of the side-chain of prednisolone with no adsorption.

The blue tetrazolium procedure as originally presented by Mader and Buck (1) required ethanolic solutions of the steroid. The modified procedure described below was devised to accommodate the aqueous steroid solutions encountered in these studies. One milliliter of aqueous sample was mixed with 9.00 ml. of absolute ethanol followed by 1.00 ml. of diluted tetramethylammonium hydroxide (5 ml. of 10% aqueous tetramethylammonium hydroxide diluted to 50 ml. with absolute ethanol) and 1.00 ml. of blue tetrazolium reagent (0.5% in 95% ethanol). The solution was mixed, color development was allowed to proceed for thirty minutes in the dark, and 1.00 ml. of glacial acetic acid was added to stabilize the color. Absorbance of the resulting solution was then determined at 530 mμ *versus* a reagent blank. Under these conditions, color production in the alkaline solution was nearly maximal after thirty minutes, although it continued to develop for two hours. Upon acidification with glacial acetic acid, the color was stable for at least seventy-five minutes as reported by Nowaczynski, Goldner, and Genest (2).

DISCUSSION

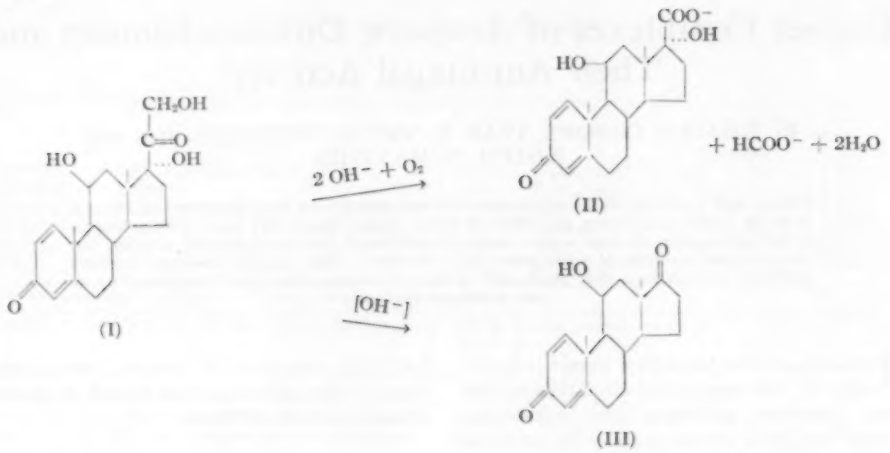
The ultraviolet analysis of the filtrates for the unsaturated carbonyl group of the steroid A-ring indicated the extent of adsorption but did not reveal degradation of the labile side-chain. On the other hand, the blue tetrazolium procedure for the α-ketol side-chain did not differentiate adsorption from degradation. Therefore, to define completely the effects encountered, the systems were analyzed by both techniques.

Typical results for the five buffers studied are presented in Table I. From the ultraviolet analyses it may be seen that adsorption occurred on the magnesium trisilicate. Agreement between the blue tetrazolium and ultraviolet analyses indicates that no degradation was involved. Similar agreement was observed between the blue tetrazolium and ultraviolet analyses of material leached from the solid magnesium trisilicate phase thereby eliminating the possibility of adsorption via degradation. The adsorption isotherm for prednisolone on magnesium trisilicate at 37.5° is shown in Fig. 1. Over the range studied the isotherm was linear and may be expressed according to the Freundlich equation $x/m = 10\,c^{1.11}$ where c is the equilibrium concentration (μg./ml.) and x/m is the amount adsorbed (μg./Gm.).

TABLE I.—EFFECTS OF SOLID BUFFERING AGENTS ON PREDNISOLONE

Buffer	pH of Suspension	Prednisolone, μg./ml. Initial	Equilibrium UV	Equilibrium BT
Magnesium trisilicate	8.9	53	34	33
		220	145	145
Magnesium oxide	10.4	220	223	140
Aluminum hydroxide	7.4	53	55	50
		220	219	225
Calcium carbonate	9.1	53	53	52
		220	220	220
Magnesium carbonate	9.2	220	222	225

Comparison of the ultraviolet and blue tetrazolium analyses in the case of magnesium oxide (Table I) shows that there was no adsorption of steroid but there was extensive side-chain degradation. From Fig. 2 it may be seen that the degradation was first order in steroid and that the pseudo first order constant (k') was independent of the initial steroid concentration and of the steroid-buffer ratio. The high pH of the magnesium oxide suspension (Table I) suggests that the degradation was due to the high alkalinity of the solution. Velluz, Petit, Pesez, and Berret (3) found that, in the presence of air and alkali, the C_{20}-C_{21} bond of the α-ketol side-chain was split with the production of the corresponding etio acid and formic acid and with the consumption of two moles of base and one mole of oxygen. This was confirmed by Herzig and Ehrenstein (4). Paper chromatographic examination of a reaction mixture obtained with magnesium oxide in the present studies gave similar results. Prednisolone (I) was converted predominantly to 3-keto-11β,17α-dihydroxy-$\Delta^{1,4}$-etiocholenic acid (II) and to a lesser degree to $\Delta^{1,4}$-androstadien-11β-ol-3,17-dione (III). Removal of the steroid side chain to give the corresponding 17-ketosteroid (III) has been reported by Mason (5) and by Wendler and Graber (6) and represents a hydroxide ion catalyzed dealdolization occurring under both aerobic and anaerobic conditions



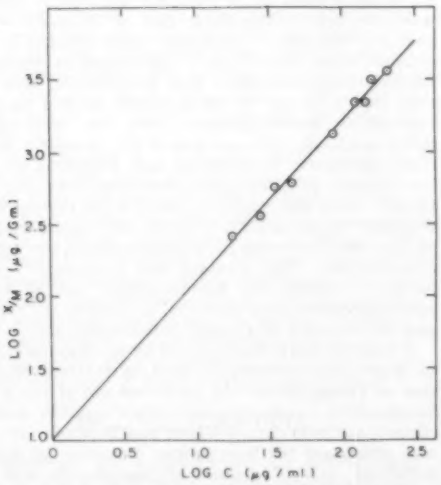


Fig. 1.—Adsorption isotherm for prednisolone on magnesium trisilicate at 37.5°

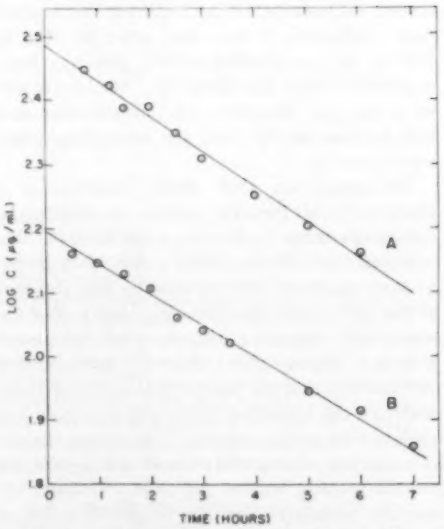


Fig. 2.—First order plots of the degradation of prednisolone by magnesium oxide suspensions at 37.5°: A, initial concentration 310 μg./ml., $k' = 127 \times 10^{-3}$ hr.$^{-1}$; B, initial concentration 155 μg./ml., $k' = 114 \times 10^{-3}$ hr.$^{-1}$.

Under the conditions employed, aluminum hydroxide, calcium carbonate, and magnesium carbonate were without effect on prednisolone (Table I).

It may be concluded therefore that the cortical steroids similar to prednisolone should not be exposed to materials capable of producing an elevated pH during formulation.

SUMMARY

The effects of aqueous suspensions of magnesium trisilicate, magnesium oxide, aluminum hydroxide, calcium carbonate, and magnesium carbonate on prednisolone at 37.5° have been studied. Magnesium trisilicate adsorbed the intact steroid. Magnesium oxide produced an alkaline degradation of the steroid side-chain. The other buffers were without effect.

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Copper Complexes of Aromatic Dithiocarbamates and Their Antifungal Activity*

By WILLIAM O. FOYE, IVAN B. VAN de WORKEEN, Jr.,† and JOSEPH D. MATTHES

Phenyl and 2-pyridyl dithiocarbamate salts and esters have been prepared and found to form stable complexes, probably chelates, with copper (II) ion. The aromatic dithiocarbamates showed a low order of antifungal and antibacterial activity, but their copper complexes were practically inactive. This finding does not support previous postulations that antifungal dithiocarbamates exert their fungitoxicity as toxic copper chelates.

INVESTIGATIONS at the duPont Company in 1931 showed that derivatives of dithiocarbamic acid possessed antifungal and antibacterial properties, and a patent reporting the antifungal activity of thiuram disulfides was issued to Tisdale and Williams (1) in 1934. The activity of several dithiocarbamic acid derivatives against fungi pathogenic to man was noted by Hall in 1938 in an unpublished report, and was later studied by Miller and Elson (2). Within twenty years of their discovery, the dithiocarbamates had become widely used for controlling plant pathogens (3).

The suggestion that metal chelation is a mechanism of biotoxic action for antifungal agents was made by Zentmyer (4) in 1943. He observed that the fungicidal action of oxine (8-hydroxyquinoline) was reversed by zinc ion, and at low pH, where the chelating ability of oxine was weak, the oxine showed weak fungicidal activity. Shortly after, Albert (5) demonstrated conclusively that the bactericidal action of oxine is due to the formation in the cell of a toxic 1:1 chelate with iron or copper. The copper chelate of oxine has subsequently found commercial use mildew-proofing textiles and paint. Goksøyr (6) has also postulated that the fungicidal action of sodium dimethyldithiocarbamate is due to the formation of toxic 1:1 chelates with copper and zinc.

To test further the hypothesis that formation of toxic metal chelates is a mechanism of fungicidal action, a series of dithiocarbamate salts and esters has been prepared in order to compare the activities of chelated and nonchelated derivatives. Aromatic dithiocarbamates were selected for this purpose and also to determine the relative fungicidal properties of aromatic dithiocarbamates, which have not been looked at to any extent for fungicidal effects.

* Received February 13, 1958, from the Massachusetts College of Pharmacy, Boston.
Abstracted from the thesis submitted by I. B. Van de Workeen, Jr., as a requirement for the degree of Master of Science, June 1956.
† Present address: Chemistry Department, Boston University, Mass.
The authors are indebted to Drs. K. K. Chen and D. Fleming of the Lilly Research Laboratories for conducting the antimicrobial tests and furnishing the results.

METHODS OF PREPARATION

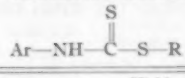
The ammonium salt of phenyldithiocarbamic acid was easily prepared by the method of Dains, Brewster, and Olander (7), and esters were obtained by standard procedures. Use of this method to obtain 2-pyridyldithiocarbamates was unsuccessful, however, and a review of the literature showed that heterocyclic dithiocarbmates have not received much attention. In a review of the chemistry of dithiocarbamates by Chabrier and Nachmias (8), for instance, no heterocyclic derivatives were mentioned. Only two references found in the literature described the preparation of pyridyl derivatives, and in only one of these was the product isolated, but not characterized. This product was a mixture believed to contain the 4-aminopyridyl salt of 4-pyridyldithiocarbamic acid (9), in analogy to the general formation of aliphatic dithiocarbamates.

Among the methods attempted for the preparation of 2-pyridyldithiocarbamic acid derivatives was that of Camps (9) for the suspected salt of the 4-isomer, using 2-aminopyridine, carbon disulfide, and ethanol, and also that of Klarer and Behnisch (10), who mentioned the intermediate formation of the potassium salt of 2-pyridyldithiocarbamic acid. Both of these methods failed, as did two procedures in which aniline has been used to obtain phenyldithiocarbamic acid. The latter two required the use of alcoholic potassium hydroxide or aqueous ammonia in the presence of carbon disulfide and the aromatic amine.

The desired reaction was accomplished by first forming the sodium salt of 2-aminopyridine in nonaqueous media with sodamide, according to Chichibabin (11), and the isolated sodium salt was then suspended in benzene and allowed to react with carbon disulfide. The product was found by analysis to be the 2-aminopyridyl salt of 2-pyridyldithiocarbamic acid, which is analogous to the product described by Camps (9) from 4-aminopyridine. Esters of this acid, which were obtained by reaction of the salt with organic halides, were difficult to purify but did give nitrogen analyses approximating theoretical. The dithiocarbamate salts and esters prepared and their properties are listed in Table I.

The reaction of copper ion with the salts and esters described above was carried out to determine the

TABLE I.—AROMATIC DITHIOCARBAMATES

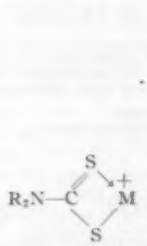


Ar	R	M. P.[a]	Yield, %	Formula	Analysis, %[b] Calcd.	Found
					C:49.98	50.41
2-Pyridyl	2-Pyridylammonium	...	86	$C_{11}H_{12}N_4S_2$	H: 4.60	5.27
2-Pyridyl	Ethyl	Oil	50	$C_8H_{10}N_2S_2$	N:14.14	13.87
2-Pyridyl	*p*-Nitrophenyl	128–130°	10	$C_{12}H_9N_3O_2S_2$	N:14.18	13.67
Phenyl	Ammonium	92–93°	73	$C_7H_{10}N_2S_2$	N:15.06	14.80
Phenyl	Ethyl	58–60°	41	$C_9H_{11}NS_2$	N: 7.11	7.21
Phenyl	*p*-Nitrophenyl	88–90°	50	$C_{13}H_{10}N_2O_2S_2$	N: 9.62	9.48

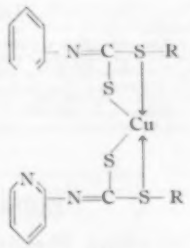
[a] The melting points were taken on a Fisher-Johns block and are uncorrected.
[b] The carbon-hydrogen analysis was obtained from the Weiler and Strauss Microanalytical Laboratory, Oxford, England. The nitrogen analyses were done by the Kjeldahl procedure, using selenized Hengar granules as catalyst.

possibility of complex or chelate formation. This was done in a suitable solvent with aqueous copper sulfate, and in each case complexing was indicated by a marked drop in pH, and formation of a colored precipitate in the case of the salts. Copper complexes soluble in the organic media used were obtained from the esters. Analysis indicated that complexes having a 2:1 ratio of dithiocarbamic acid to copper were obtained from the salts and the *p*-nitrophenyl esters, and complexes with a 1:1 ratio were obtained from the ethyl esters. The physical properties of the complexes prepared are listed in Table II.

The structures of 1:1 metal chelates of dithiocarbamic acids have been determined by Chatt, *et al.* (12), and pictured as four-membered rings (structure I) with several resonating forms possible. The 2:1 copper complexes can accordingly be represented by structure II, although open-chain complexes with the copper coordinated to two sulfurs or possibly two nitrogens are also possible and would also cause a drop in pH on formation. Complexing by means of the pyridine nitrogen is not postulated for these compounds, since the phenyl derivatives gave the same results as the pyridyl.



I



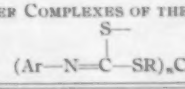
II

BIOLOGICAL RESULTS

Antifungal and antibacterial tests were carried out at the Lilly Research Laboratories, and the results of positive activity are shown in Table III. It is apparent that the salts of phenyl- and 2-pyridyldithiocarbamic acids have essentially the same activity against microorganisms; while the degree of activity differed slightly in several cases, these compounds generally showed activity toward the same organisms, and both were also negative toward a number of other organisms.

The copper chelates of these acids, on the other hand, showed essentially no activity toward the test organisms. Only *U. avenae* was inhibited by the chelates at a concentration of 200 mcg./ml. but not at 50 mcg./ml. (The esters and their copper derivatives have not as yet been tested.) This result implies that dithiocarbamates do not exert their fungicidal action in the form of toxic copper chelates, although chelation of essential copper in the organisms is still a plausible mechanism of action. Further such comparisons should be made with other dithiocarbamates, however, although the comparative results might be obscured somewhat with weaker chelators or stronger antifungal agents.

TABLE II.—COPPER COMPLEXES OF THE DITHIOCARBAMATES



Ar	R	*n*	pH Drop[a] pH Units	Yield, %	M. P.[b]	Color	Formula	N Analysis, %[c] Calcd.	Found
2-Pyridyl	H	2	6.6	74	115°(dec.)	Green-black	$C_{12}H_{10}N_4S_4Cu$	13.93	13.42
2-Pyridyl	C_2H_5	1	3.4	40	88–90°	Green-black	$C_8H_{10}N_2S_2Cu$	9.40	9.94
2-Pyridyl	$C_6H_4NO_2$	2	2.0	48	132–134°	Yellow-green	$C_{24}H_{16}N_6O_4S_4Cu$	12.96	11.76
Phenyl	H	2	3.0	84	110°(dec.)	Yellow	$C_{14}H_{12}N_2S_4Cu$	7.00	6.96
Phenyl	C_2H_5	1	3.0	56	50–52°	Yellow-green	$C_9H_{11}NS_2Cu.2H_2O$	4.71	5.10
Phenyl	$C_6H_4NO_2$	2	2.5	50	103–105°	Red	$C_{26}H_{18}N_4O_4S_4Cu$	8.67	8.53

[a] The drop in pH was recorded during the addition of copper sulfate.
[b] The melting points were taken on a Fisher-Johns block and are uncorrected.
[c] The nitrogen analyses were done by the Kjeldahl procedure, using selenized Hengar granules as catalyst.

TABLE III.—ANTIMICROBIAL TESTING OF THE DITHIOCARBAMATE SALTS[a]

Test Organism	Inhibitory Concn., mcg./ml.[b] Phenyl Deriv.[c]	2-Pyridyl Deriv.[d]
Staphylococcus albus	200	200
Bacillus subtilis	50	50
Mycobacterium tuberculosis	50	50
Klebsiella pneumoniae	100	...
Trichophyton interdigitale	100	100
Shigella paradysenteriae	...	100
Xanthomonas phaseoli	25	25
Monilinia fructicola	100	50
Ustilago avenae	12.5	12.5
Alternaria solani	<6.25	<6.25
Fusarium moniliforme	200	...
Colletotrichum gossypii	50	100
Ascochyta imperfecta	25	200
Sclerotinia bataticola	50	50

[a] Carried out at the Lilly Research Laboratories by D. Fleming.
[b] The agar dilution technique was used, the bacteria being observed for forty-eight hours, and the bacterial and fungal plant pathogens being observed for seventy-two hours. Those organisms having no inhibitory levels indicated were not affected by 200 mcg./ml.
[c] Ammonium phenyldithiocarbamate.
[d] 2-Pyridylammonium 2-pyridyldithiocarbamate.

EXPERIMENTAL

Ammonium Phenyldithiocarbamate.—The procedure of Dains, Brewster, and Olander (7) was used. A 73% yield of white powder was obtained which melted at 92–93°. Losanitsch (13) first described this compound as decomposing at 100°. Dains, *et al.*, list no physical constants.

Ethyl Phenyldithiocarbamate.—Three grams (0.016 mole) of ammonium phenyldithiocarbamate and 75 ml. (1 mole) of ethyl bromide were stirred at room temperature for four hours. The mixture was filtered, and the filtrate was distilled under reduced pressure. The remaining semisolid was taken up in ether and allowed to crystallize, giving 1.25 Gm. (41%) of yellow-green crystals; m. p. 58–60°.

***p*-Nitrophenyl Phenyldithiocarbamate.**—One gram (0.005 mole) of ammonium phenyldithiocarbamate was dissolved with gentle heating in 30 ml. of distilled water. Ethyl alcohol (20 ml.) and *p*-nitrochlorobenzene (1.0 Gm., 0.006 mole) were added, and the solution was refluxed for one hour. A light yellow powder precipitated after the solution cooled, giving 0.8 Gm. (50%) of product melting at 88–90°.

2-Pyridylammonium 2-Pyridyldithiocarbamate.—A solution of 12 Gm. (0.31 mole) of sodamide in 50 ml. of benzene was treated dropwise with 25 Gm. (0.27 mole) of 2-aminopyridine dissolved in 175 ml. of benzene with warming. The mixture was refluxed for two hours and allowed to cool. A solution of 17 ml. (0.28 mole) of carbon disulfide in 50 ml. of benzene was then added dropwise with stirring, and the resulting solution was refluxed with stirring (mercury seal) for four hours and allowed to stand overnight. The yellow precipitate which appeared was washed with ether and dried. The yield was 30 Gm. (86%). Erratic results were obtained from Kjeldahl determinations on this product using either selenium or mercuric oxide as catalyst, but a carbon-hydrogen combustion analysis gave values approaching theoretical. The product was contaminated with sodium ion, however.

Ethyl 2-Pyridyldithiocarbamate.—Three grams (0.011 mole) of the 2-pyridyldithiocarbamate salt and 75 ml. (1 mole) of ethyl bromide were stirred at room temperature for four hours. The mixture was filtered, and the filtrate was distilled under reduced pressure. The red, oily liquid remaining was dissolved in ether, which was partially evaporated. A yield of 1.5 Gm. (68%) of red oil was obtained which decomposed on attempted distillation under reduced pressure.

***p*-Nitrophenyl 2-Pyridyldithiocarbamate.**—One gram (0.005 mole) of the 2-pyridyldithiocarbamate salt was dissolved with gentle heating in 30 ml. of distilled water. Ethyl alcohol (20 ml.) and *p*-nitrochlorobenzene (10 Gm., 0.006 mole) were added, and the solution was refluxed for one hour. A green powder precipitated after the solution cooled, giving 0.15 Gm. (10%) of product melting at 128–130°.

Chelation with Copper (II) Ion.—The following procedure is representative. To a filtered solution of 10 Gm. (0.05 mole) of ammonium phenyldithiocarbamate in 75 ml. of water was added gradually, with stirring, a solution of 4 Gm. (0.025 mole) of copper sulfate in 50 ml. of water. During the reaction the pH dropped from 8.3 to 5.3. A yellow precipitate was collected, washed with water, and allowed to dry at room temperature. The yield was 9.0 Gm. (84%); and the product decomposed at 110°.

In the corresponding reaction with the esters, an organic solvent was used—acetone for the ethyl esters and ethanol for the *p*-nitrophenyl esters. After treatment with aqueous copper sulfate solution in which a minimum amount of water was used, the unreacted copper sulfate was removed by filtration. The filtrate was then allowed to evaporate to dryness to yield the complexes.

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Chemical Investigation of *Daemia extensa* R. Br.*

By S. PATTABI RAMAN and A. K. BARUA

A chemical study of *Daemia extensa* is described. From the petroleum ether extract of the whole plant, three triterpene compounds and a sterol have been isolated and characterized.

DAEMIA EXTENSA R. Br. (syn. *Pergularia extensa* N. E. Br.) belongs to the family *Asclepiadaceae* and it grows wildly in India. It is extensively used in the indigenous system of medicine in India (1). From the above plant, Gupta, *et al.* (2), described a pharmacologically active glucosidic bitter principle having action on uterus comparable with that of pituitrin. Dutta and Ghosh (3) reported the isolation of four sterols having m. p.'s 172–76°, 163°, 157°, and 70–80°, respectively, from the same plant. The bitter principles could not, however, be obtained in a pure state, nor were the sterols characterized by them. In view of the important physiological action of the drug, a systematic chemical investigation of the whole plant has been undertaken by us.

The petroleum ether extract of the plant on chromatographic resolution over Brockmann's alumina gave three triterpene compounds, α-amyrin acetate, lupeol acetate, and lupeol and also a sterol which could be identified as β-sitosterol. It has not yet been possible to crystallize the bitter fractions obtained from the benzene, chloroform, and alcoholic extracts of the plant and attempts are being made for the same.

EXPERIMENTAL

Air-dried powdered drug (whole plant, 700 Gm.) was Soxhleted with petroleum ether (b. p. 60–80°). The volume of the extract was reduced to about 100 cc. and was adsorbed on a column of Brockmann's alumina (45 cm. x 2 cm.). The column was then eluted successively with petroleum ether (b. p. 60–80°, 1,000 cc.), petroleum ether-benzene mixture (1:1,600 cc.), benzene (400 cc.), benzene-chloroform mixture (1:1 1,200 cc.), and finally with methanol. The petroleum ether eluate, on removal of the solvent and crystallization of the residue from chloroform-methanol mixture, gave almost white amorphous solids (5.2 Gm., Fraction A). The petroleum ether-benzene and benzene eluates, on removal of the solvent, left residues which on crystallization from aqueous ethanol gave colorless crystalline compounds of the same m. p., 152–164° (0.8 Gm. total, Fraction B). The benzene-chloroform and chloroform eluates gave residues which on crystallization from aqueous ethanol gave almost colorless flakes of the same m. p., 131–135° (1 Gm. total, Fraction C). The methanol eluate left negligible amount of residue.

Fraction A was taken up in benzene (15 cc.) and was adsorbed on a column of Brockmann's alumina (16 cm. x 3 cm.) and was eluted with petroleum ether (b. p. 40–60°, 800 cc.), collecting 25-cc. portions. The first ten fractions yielded a white amorphous mass (3 Gm., m. p. 115–137°, Fraction D) whereas the subsequent fractions had the extended m. p. 140–165° (1.2 Gm., Fraction E). The latter fraction on repeated crystallization from chloroform-methanol mixture gave colorless needles, m. p. 213–215°, $[\alpha]_D$ + 47.8° (in chloroform) which did not depress the melting point of an authentic sample of lupeol acetate.

Anal.—Calcd. for $C_{32}H_{52}O_2$: C, 81.99%; H, 11.12%. Found: C, 81.96%; H, 11.3%.

The above product on hydrolysis with ethanolic caustic potash (10%) gave colorless needles from ethanol, m. p. 211–212°, which depressed the m. p. of the parent compound but not that of lupeol.

Anal.—Calcd. for $C_{30}H_{50}O$: C, 84.44%; H, 11.81%. Found: C, 84.32%; H, 11.9%.

It gave a benzoate, m. p. 263–264°, which was found to be identical with lupeol benzoate by mixed m. p. determination.

Anal.—Calcd. for $C_{37}H_{54}O_2$: C, 83.7%; H, 10.3%. Found: C, 83.69%; H, 10.22%.

Fraction D on rechromatography and crystallization from chloroform-methanol gave shining leaflets, m. p. 222–223°; $[\alpha]_D^{33}$ + 79° (in chloroform), which showed no change in melting point when admixed with an authentic sample of α-amyrin acetate.

Anal.—Calcd. for $C_{32}H_{52}O_2$: C, 81.99%; H, 11.12%. Found: C, 81.95%; H, 11.25%.

On hydrolysis with ethanolic caustic potash (10%) it gave a compound m. p. 184–185°, crystallizing as needles from ethanol, which was shown to be identical with α-amyrin by mixed m. p. determination.

Anal.—Calcd. for $C_{30}H_{50}O$: C, 84.44%; H, 11.81%. Found: C, 84.3%; H, 11.9%.

This gave a benzoate m.p. 194–196°, identical with α-amyrin benzoate.

Anal.—Calcd. for $C_{37}H_{54}O_2$: C, 83.7%; H, 10.3%. Found: C, 83.81%; H, 10.38%.

Fraction B was directly benzoylated and chromatographed over active alumina when a fraction, m. p. 261–262°, was obtained in major yield. This was shown to be lupeol benzoate by mixed m. p. determination.

Anal.—Calcd. for $C_{37}H_{54}O_2$: C, 83.7%; H, 10.3%. Found: C, 83.69%; H, 10.22%.

This on hydrolysis gave lupeol. Thus fraction B is shown to contain lupeol in the free state.

* Received February 24, 1958, from the Department of Chemistry, Bose Institute, Calcutta, India.

The authors are indebted to Dr. D. M. Bose, Director of this Institute and to Dr. P. K. Bose, Head of the Department of Chemistry for their keen interest in this work. Thanks also are due to Mrs. C. Dutta, University College of Science & Technology, Calcutta, for microanalysis of the compounds reported in this paper and also to the Government of India, Ministry of Education, for the award of a National Research Fellowship to one of them (A. K. B.).

Fraction C on repeated crystallization from aqueous ethanol gave shining leaflets, m. p. 136–137°, $[\alpha]_D^{32}$ − 35.5° (in chloroform). Admixture with an authentic sample of β-sitosterol showed no change in m. p.

Anal.—Calcd. for $C_{29}H_{50}O$: C, 83.99%; H, 12.15%. Found: C, 83.71%; H, 12.0%.

With pyridine and acetic anhydride at 100°, it gave an acetate m. p. 127–128°, $[\alpha]_D^{32}$ − 41.5° (in chloroform).

Anal.—Calcd. for $C_{31}H_{52}O_2$: C, 81.52); H, 11.44%. Found: C, 81.6%; H, 11.51%.

The sterol also furnished a benzoate by the usual method, m. p. 143–144°, which did not depress the m. p. of an authentic sample of β-sitosterol benzoate.

Anal.—Calcd. for $C_{36}H_{54}O_2$: C, 83.34%; H, 10.49%. Found: C, 83.32%; H, 10.35%.

SUMMARY AND CONCLUSIONS

The petroleum ether extract of *Daemia extensa* R. Br., has been found to contain α-amyrin acetate, lupeol acetate, lupeol, and β-sitosterol. It appears that the sterols obtained by the previous workers were not homogeneous compounds but mixtures of the above substances.

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Bromide Intoxication II. Biochemical and Hematological Aspects*

By IRA ROSENBLUM and THOMAS L. HAWKINS, Jr.

Progressive bromide intoxication in dogs was shown to be accompanied by a decrease in the volume of the extracellular space and in the terminal stages by hemoconcentration. Mild and transient anemia and leukocytosis occurred in a number of dogs and the sedimentation rate was accelerated in almost all the animals.

DESPITE the fact that chronic bromide intoxication is regarded as being largely neurointoxication, there is little evidence which excludes the possibility that other systems are affected. In a comprehensive review of the clinical literature of bromide intoxication through 1940 (1) there was no mention of the state of liver and kidney function, electrolyte levels, acid-base equilibrium, etc. Since then, Clark, *et al.* (2), have examined the effects of small doses of bromide on some of these systems in animals. Experiments carried out in this laboratory (3) have demonstrated that dogs intoxicated chronically with varying doses of bromide become emaciated, lose weight, and otherwise show signs of systemic dysfunction although signs of neurointoxication are absent. This would support the possibility that bromide disturbs the function of systems other than the central nervous system. The present experiments were performed to assess the function of some of these systems during chronic bromide intoxication.

* Received February 24, 1958, from the Department of Pharmacology and the Sub-Department of Hematology, Albany Medical College, Albany, N. Y.

The technical assistance of Mrs. Norma Hurst is gratefully acknowledged.

Supported by a grant from The Institute for the Study of Analgesic and Sedative Drugs.

METHODS

Adult mongrel dogs of both sexes were used. These animals were treated with a standard veterinary vermifuge and inoculated against canine distemper and hepatitis prior to the start of the experiments. They were kept in individual metabolism cages during the entire experimental period and maintained on a diet of commercial dog food, 40 Gm./Kg. of body weight per day, with water available at all times.

Because each dog was to serve as its own control in these experiments, biochemical and hematological measurements were made at least twice on each dog before administration of bromide was begun. The course of experimental bromide intoxication in dogs produces signs which resemble those seen in man. These have been reported in detail elsewhere (3). In these experiments two procedures were used to produce bromide intoxication. In one group of 4 dogs, intoxication was produced rapidly by administering an initial dose of 400 mg. of sodium bromide per Kg. of body weight per day. This dose was increased once after three weeks to 500 mg. of sodium bromide per Kg. of body weight per day. These dogs were used in one hematological experiment. Death occurred rapidly in this group and all were dead by the end of the sixth week of administration of sodium bromide. Slow intoxication was produced in three groups, each of four dogs, by giving 200 mg. of sodium bromide per Kg. of body weight per day initially. This dose was in-

creased every six weeks by 100 mg. of sodium bromide per Kg. of body weight per day until one of the animals died. The maximum dose of sodium bromide which was reached by this procedure was 600 mg. per Kg. of body weight per day. These groups were used in both the biochemical and hematological experiments.

Biochemical Methods.—Blood glucose was determined by the method of Nelson and Somogyi using zinc sulfate-barium hydroxide to deproteinize the blood. Urea was converted to ammonia with urease and the nitrogen determined spectrophotometrically using Nessler's reagent. The CO_2 combining power of serum was measured manometrically by the method of Peters and Van Slyke.

Bromsulfalein retention was measured forty-five minutes after injection of the dye. Serum Na and K were measured using a Beckman flame photometer with an internal lithium standard. References to these foregoing procedures may be found in Hawk, Oser, and Summerson (4). Serum inorganic phosphorus was measured by the method of Gomori (5), plasma volume was measured by the method of Gibson and Evans (6), and the thiocyanate space was measured by the method of Gregerson and Stewart (7) using Bowler's method for determining thiocyanate (8). Serum calcium was determined by the method of Kibrich, *et al.* (9), and blood bromide was determined by the method of Brodie and Friedman (10). Urinary coproporphyrins were measured by the fluorometric method of Schwartz, *et al.* (11).

Hematological Methods.—Measurements were made on blood drawn from the jugular vein using heparin as an anticoagulant. Single erythrocyte and leukocyte counts were made using one blood pipet and the Neubauer hemocytometer. Differential leukocyte counts were made on slides stained by Wright solution. Reticulocyte counts were made by the "dry" method using brilliant cresyl blue as the stain. Hemoglobin determinations were made with the Fisher Electro-Hemometer. Hematocrit and "corrected" sedimentation rate were measured by Wintrobe's method (12). Bone marrow aspirations were made with an 18-gauge needle from a spinous process and were processed according to Propp's method (13).

RESULTS

Biochemical.—The biochemical values of the control period are compared to those at seven and twenty weeks after administration of sodium bromide in Table I. Thereafter measurements were obtained from only one dog in each group and so could not serve as a basis for comparison with the control values.

An important sign of bromide intoxication in dogs is the appearance of emaciation and weight loss on undiminished food intake. The effect occurs in most dogs at doses of bromide which cause no other signs of toxicity. One cause for this change appears to be a reduction in the volume of the extracellular space. In this group of dogs, the mean control value for the extracellular (thiocyanate) space was 322 ± 39 ml./Kg.; after the seventh week of ingestion of bromide it had fallen to 225 ± 27 ml./Kg. The plasma volume did not however change significantly during this time and the serum electrolytes were likewise unaltered. Changes in plasma volume and serum sodium and potassium do sometimes occur during bromide intoxication, especially during the terminal stages when the animals are no longer able to eat or drink. At that time, serum sodium and potassium are elevated, probably due to hemoconcentration since the plasma volume is reduced and the hematocrit values increased.

TABLE I.—AVERAGE BIOCHEMICAL VALUES IN DOGS BEFORE AND AFTER ADMINISTRATION OF SODIUM BROMIDE (SLOW INTOXICATION)

	Initial	7 Wk.[b,c]
No. of Dogs	4	3
Blood Br, meq./L.	0.7 ± 0.1[a]	52.8 ± 5.6[a]
Serum Na, meq./L.	147 ± 1	142 ± 1
Serum K, meq./L.	4.1 ± 0.1	4.4 ± 0.1
Plasma vol., ml./Kg.	42.2 ± 3.8	45.0 ± 4.7
SCN space, ml./Kg.	322 ± 39	225 ± 27

[a] Standard deviation of mean.
[b] Only one dog survived after the seventh week.
[c] The dose of NaBr at this time was 300 mg./Kg. of body weight per day.

Emaciation may also be the result of derangements in metabolism. The liver and kidney are important organs which are concerned with the metabolism of protein and carbohydrate so that dysfunction of these organs might well lead to emaciation and weight loss. Several functions of these organs were studied in a group of four dogs. These studies indicate that neither bromsulfalein retention nor blood urea nitrogen were changed significantly during bromide intoxications. For example, the mean control value for bromsulfalein retention was 4 ± 3% and after twenty weeks of ingestion of sodium bromide it was 3 ± 2%. The mean control blood urea nitrogen was 16.7 ± 2.1 mg./100 ml. and after twenty weeks of ingestion of sodium bromide it was 14.7 ± 2.9 mg./100 ml.

It is also evident that these organs were able to contribute to the maintenance of the blood glucose at about the control level (i. e., control blood glucose, 84 ± 10 mg./100 ml., after twenty weeks, 80 ± 10 mg./100 ml.). Other values which were essentially unchanged after twenty weeks of ingestion of sodium bromide were serum calcium, serum phosphorus, serum carbon dioxide combining power, and total urinary coproporphyrins.

Hematological.—The hematological values of the control period are compared to those at seven and nineteen weeks after administration of sodium bromide in Table II, in dogs which underwent slow intoxication. There was little difference between the responses of those dogs subjected to rapid intoxication and those slowly intoxicated and they will be considered together.

There is little definite information in the literature about the status of the hematopoietic system during bromide intoxication. When high blood bromide levels are encountered in cases of human bromide intoxication, the changes observed in the hematopoietic system are equivocal (14). However it has been suggested that both mild anemia and

TABLE II.—AVERAGE HEMATOLOGICAL VALUES IN DOGS BEFORE AND AFTER ADMINISTRATION OF SODIUM BROMIDE (SLOW INTOXICATION)

	Group 1		Group 2	
	Initial	7 Wk.[b,d]	Initial	19 Wk.[c,e]
No. of dogs	4	3	4	3
Blood Br, meq./L.	0.8 ± 0.1[a]	52.8 ± 5.6[a]	0.7 ± 0.1[a]	52.3 ± 9.2[a]
Erythrocytes × 10^6/cu. mm.	5.8 ± 0.5	5.0 ± 1.3	6.8 ± 0.7	6.1 ± 0.1
Reticulocytes, %	0.4 ± 0.3	0.8 ± 0.5		
Hemoglobin, Gm./100 ml.	11.8 ± 0.6	11.1 ± 1.9	14.8 ± 1.1	13.1 ± 0.6
Hematocrit, %	41 ± 3	32 ± 6	47 ± 5	39 ± 1
Leukocytes × 10^3/cu. mm.	15.6 ± 3.6	25.1 ± 17.0	12.5 ± 1.9	14.2 ± 3.1
Band Neutrophils, %	8 ± 2	11 ± 7	8 ± 4	5 ± 1
Mat. Neutrophils, %	68 ± 4	71 ± 1	65 ± 3	71 ± 6
Lymphocytes, %	16 ± 5	12 ± 5	15 ± 5	14 ± 6
Monocytes, %	2 ± 1	5 ± 2	8 ± 5	5 ± 1
Eosinophils, %	5 ± 4	5 ± 2	5 ± 4	6 ± 1
Sedimentation Rate, mm./hour	4 ± 1	31 ± 11	8 ± 5	23 ± 1

[a] Standard deviation of mean.
[b] Only one dog survived after the seventh week.
[c] Only two dogs survived after the nineteenth week.
[d] The dose of NaBr at this time was 300 mg./Kg. of body weight per day.
[e] The dose of NaBr at this time was 500 mg./Kg. of body weight per day.

transitory leucocytosis does occur (14), and our observations would seem to bear this out.

Erythrocytes.—Some decrease in erythrocytes was noted in dogs in both groups during the course of intoxication. These animals showed concomitant decreases in hemoglobin and hematocrit values. The greatest loss of hemoglobin was approximately 6 Gm./100 ml. which occurred in a dog in group two, fourteen weeks after administration of sodium bromide. This dog's hemoglobin value gradually rose toward the control value by the nineteenth week. Two dogs in group one showed losses of approximately 2 Gm./100 ml. of hemoglobin six weeks after administration of sodium bromide was begun. However, the anemias noted in most of the animals were not marked.

Leukocytosis.—Two of the dogs in group one showed striking increases in total leukocyte counts. These dogs showed counts of 83,000 and 53,000, four weeks after administration of sodium bromide was begun. One of them died at the end of the fifth week; the other continued to show an elevated leukocyte count at the seventh week and it died on the eighth week. Three of the dogs in group two developed leucocytosis of a milder degree but this decreased to approximately the control levels by the nineteenth week. There was little change in the differential leukocyte counts in any of the dogs and no early cell forms were seen.

Bone Marrow.—Studies were made of the bone marrow of the dogs in group 2. Twenty-eight examinations were made in all, both before and during administration of sodium bromide. The interpretation was based on inspection of all the material obtained; no differential counts were done. The Granulocytic/Erythrocytic ratios (G/E) of 3 dogs varied from 1/1 to 3/1. One dog persistently had ratios of 4/1 to 6/1. There was no significant change in cellularity or G/E ratio during the course of intoxication. No increases were noted in blast forms or defects in maturation. Megakaryocytes remained normal in number and appearance.

Sedimentation Rate.—Accelerated sedimentation rates were noted in all animals following administration of sodium bromide. These remained elevated throughout the course of intoxication. Such changes were seen even in those dogs which showed no signs of anemia and so were probably not caused by changes in the size and hemoglobin content of the erythrocytes. These findings in dogs are in agreement with those of Dax (15) who reported accelerated sedimentation rates in 19 of 59 cases of human bromide intoxication. Since the sedimentation rate is influenced by the composition of the plasma proteins, acceleration of the rate may indicate that an imbalance in plasma protein fractions occurs during bromide intoxication.

SUMMARY

1. The progressive intoxication of dogs with sodium bromide results in a striking decrease in the volume of the extracellular space. In the terminal stages of intoxication, plasma volume may be decreased and serum sodium and potassium elevated as a result of hemoconcentration.

2. Both mild and transitory anemia and leucocytosis was observed in a number of dogs. Severe leukocytosis developed in only two dogs. Acceleration of the sedimentation rate occurred in most dogs during bromide intoxication.

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Synthesis of Epoxide Polymers of Steroidal Compounds*

By W. F. HEAD, JR.,† and W. M. LAUTER

A series of twenty ethylene oxide and propylene oxide addition polymers of ursolic acid and some of its derivatives have been made and characterized. In addition, an ethylene oxide and propylene oxide polymer of cholic acid and ethylene oxide polymers of vitamin A, vitamin D_3, vitamin E, cortisone, estradiol, and testosterone are reported. Attempts to prepare steroidal polymers with highly branched or cross-linked side chains by using glycidol and epichlorhydrin were unsuccessful. The most acceptable structures as determined from molecular weight and solubility data were determined. Percentage polyalkylene oxide content, solubilities, and molecular weight or chain length distributions are presented.

URSOLIC ACID, its alkali, alkaline earth, and other metallic salts have been examined for possible use as emulsifiers in various emulsion, ointment, and cosmetic formulations (1, 2); however, none of these products offers serious competition to the presently available surface-active agents used for these purposes. The effectiveness of these salts is probably hindered by their extreme hydrophobic character. One of the aims of this investigation was to extend the work previously done in the light of some of the newer concepts of the chemistry of surface-active agents in the hope of obtaining a product or series of products derived from ursolic acid which are equal to or superior to those in present use.

The introduction of more hydrophilic groups into ursolic acid derivatives and similar compounds would seem to facilitate their surfactant properties by bringing about a better oil-soluble, water-soluble balance within the molecule. Hydrophilic, nonionic, polyalkylene oxide chains were utilized in this work because of their compatibility with a larger variety of materials. The number of these chains projecting from the steroidal nucleus can be varied by virtue of two reactive centers existing in ursolic acid. Consequently, the degree of hydrophilicity can be varied by the use of the proper intermediate. The intermediates were chosen so that the attachment of polyepoxide chains could be controlled in number and position, thus producing a series of compounds which would orient themselves properly at various liquid-liquid interfaces. Also, two epoxide monomers, which produce polyalkylene oxide chains with different hydrophilicities, were used. These were ethylene oxide and propylene oxide, the latter being less hydrophilic. As this work progressed, the information obtained was extended to cholic acid, which is similar to ursolic acid in structure and properties, for purposes of increasing its own inherent surface activity.

Another facet of this investigation, that of inducing water solubility in a normally insoluble compound, was also suggested by the experimental data obtained using ursolic acid. A series of polyethylene oxide polymers of various water-insoluble vitamins and hormones were prepared with the hope of producing water-soluble, physiologically active materials. In this manner, pharmaceutical formulation and dosage administration problems might be minimized.

EXPERIMENTAL

Materials.—The monomers and initiators, with the exception of ursolic acid, were all obtained from reputable manufacturers and were considered to be of sufficient purity to use without further purification.

Generous quantities of crude ursolic acid were supplied by the National Cranberry Association, Hanson, Mass. The crude material contains 25–50% ursolic acid, the remainder being composed of other triterpenoids, resins, triterpenoid acids, sterols, waxes, extraneous plant material, and coloring matter. Purification was carried out by two methods, one employing decolorization of the ammonium salt (3) and the other simply decolorization of an alcoholic solution of the free acid (4). The former method is somewhat longer but purer ursolic acid is obtained after fractional crystallization. The semipure product from the free acid decolorization is obtained quickly and is probably 90–95% ursolic acid. Since the polymers produced are mixtures themselves, this material was also considered acceptable.

From the purified ursolic acid, various compounds were made so that polyepoxide chains could be

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attached selectively. These intermediates have been described previously and are as follows: ursonic acid (5), sodium ursolate (6), calcium ursolate (7), acetyl ursolyl amide (8–10), ursolyl amide (10), methyl ursolate (11), ursolyl amine C-2 (10), and ursolyl amine C-28 (10).

Methods.—For the preparation of these polymers, the reaction of liquified epoxides with initiator (corresponding to ursolic acid, its derivatives, cholic acid, and various vitamins and hormones) and catalyst under pressure and in the absence of solvent was utilized. A stainless steel bomb with snugly fitting, glass bomb liners of approximately 125-ml. capacity, E. H. Sargent and Co. number S-66950, was used. The top of the bomb was fitted with a pressure gauge reading 0–300 psig.

In general, 0.5 Gm. to 1.0 Gm. of initiator, approximately 0.5% to 1.0% catalyst, usually crushed sodium hydroxide or potassium hydroxide pellets, and 50 ml. to 60 ml. of epoxide at −20° were mixed well in the bomb liner, inserted into the bomb and heated. In some cases, the initiator was freely soluble in the monomer even at −20°; it was felt that those initiators which were not completely soluble dissolved as the temperature was increased. The pellets of alkali catalysts were crushed to provide more catalytic surface area, although this meant the introduction of a very slightly larger quantity of moisture into the mixture. Moisture, in significant amounts, is very undesirable in these processes since epoxides react with water to produce polyepoxides at a rate highly competitive with the desired reaction. For this reason, all mixing was done as quickly as possible and the hygroscopic alkalies were not allowed to be exposed to the air any longer than necessary.

As the temperature increased, the pressure also increased and an attempt was made to find that temperature which would produce a pressure maximum of approximately 150 psig. In some cases, "peaking" occurred and pressures well in excess of 300 psig. were encountered. An indication of reaction termination was considered to be a drop in pressure without alteration of heat input. In some cases, with the more reactive initiators, a drop in pressure of 150 psi. took place within seven hours. With less reactive monomers or initiators a drop of only 10 psi. to 20 psi. was observed after forty-eight hours or even longer. This did not mean that polymeric material was not formed, rather that some monomer remained unreacted. Only a very small amount of epoxide is required to maintain a high pressure and rarely did pressures fall completely to 0 psig. without removing the source of heat.

At the termination of a reaction, excess unreacted monomer was allowed to escape from the safety valve and the bomb was opened. Products obtained were either waxy solids or semisolids or viscous, oily liquids. In most cases a slight tan coloration was imparted to the compounds and purification was considered necessary. For removal of this color, decolorization by heating a chloroform solution of the polymer mass with animal charcoal, filtration, and evaporation of the chloroform was employed.

It was considered necessary to determine if a polymer, other than a polyalkylene oxide, had been formed. This was accomplished by applying the Liebermann-Burchard test for the presence of the steroidal nucleus to the purified polymer (12). In addition, certain solubility relationships were also used. While the ethylene oxide polymers produced were completely water soluble, a physical mixture of the polyethylene glycols, corresponding in molecular weight to that of the polymer formed, and the initiator material, when dissolved in water, deposited nearly all of the initiator in the form of a white precipitate. The propylene oxide polymers were insoluble in water; however, they did not deposit initiator material as did the corresponding control tests.

A series of reactions, using both ethylene oxide and propylene oxide, was performed, in the presence of catalyst only, for varying periods up to seventy-two hours. The amount of polymer formed was weighed. It is improbable that this polymer could have been formed from any reaction other than that between the epoxide and trace amounts of water in the catalyst, producing polyalkylene oxides, since initiators were not present. From these data, curves were constructed so that the amount of polyalkylene oxide in any of the steroidal polymer masses could be approximated by observing the time length of the reaction.

Polyepoxide chains terminate at some point in a hydroxyl group. The unknown factor in this series of compounds was the number of these chains projecting from a given initiator. This was usually determined by noting the number of hydrogen atoms, available for polymerization, appearing in the initiator. This is not always true because of steric hindrance, hydrolysis during the course of a reaction, etc.

The end hydroxyl groups of all polymers were determined by the method of Siggia (13). Calculations were then based on the most likely structure for determining the molecular weight. As additional data, molecular weights were also determined using an ebullioscopic method with chloroform as the solvent (14). If these two methods gave results which were in support of each other, the structure chosen was taken to be the most acceptable. If the physical data did not agree with the chemical method, the latter was recalculated using a different number of projected polyoxyalkylene chains. This changes the number of end group hydroxyls which, in turn, causes the molecular weight value to vary when the same data used before are applied. The number of chains were not simply picked at random, but had to be compatible with the number possible using a given initiator. Recalculations were made until the two methods were in agreement, then this structure was accepted. In some cases, the polymers were polyoxyalkylene esters. Therefore, for these compounds, a third molecular weight determination was made using saponification data (15). Agreement for one structure was then required of all three methods of analysis. In this way, the most acceptable structure and molecular weight for all polymers were determined.

In order to determine the molecular weight distribution and the corresponding percentages by weight of the total polymer mass, a Poisson distribution, based on the determined average molecular weight, was calculated (16). The average number of epoxide units per polymer molecule, as

obtained from molecular weight data, is represented by ν in the equation below, where W_x is the weight percentage of a particular X-mer, and X is the number of epoxide units in this X-mer.

$$W_x = \left[\frac{\nu}{\nu + 1}\right] \frac{X\nu^{X-2}}{e^{\nu}(X-1)!}$$

A representative curve is shown in Fig. 1 for the ethylene oxide polymer of ursolic acid. The values of ν for the remainder of the polymers are reported as average chain length in Tables I and II.

Results.—The most acceptable structures for the polymers reported are the same as would be predicted from the active polymerizable hydrogen content of the respective initiators, with the exception of the ethylene oxide polymer of acetyl ursolyl amide and the propylene oxide polymer of ursolic acid.

Since the acetyl group has active hydrogen atoms, it is possible that epoxide reaction could occur at this point. In order to determine if this mechanism was taking place in the synthesis of the acetyl ursolyl amide-ethylene oxide polymer, pregnenalone acetate, a compound containing a ketonic carbonyl group and an acetate ester but no other source of available hydrogen, was chosen as a blank. A poly-

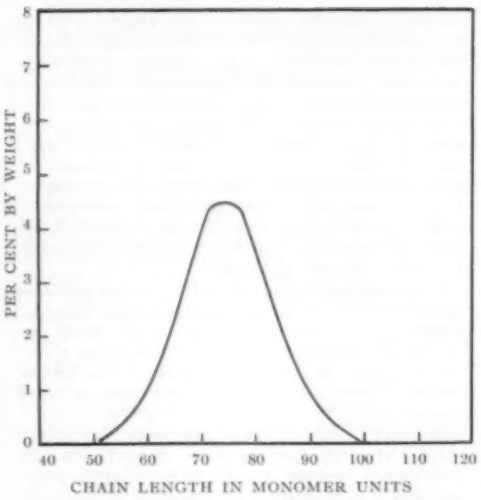

Fig. 1.—Distribution of chain length in ursolic acid–ethylene oxide polymer.

TABLE I.—REACTION CONDITIONS AND PROPERTIES OF ETHYLENE OXIDE POLYMERS

Initiator	Reaction Time, Hr.	Reaction Temp., °C.	Polyethylene-Oxide Content, %	Molecular Weight[a] A	Molecular Weight[a] B	Molecular Weight[a] C	Av. Chain Length[b]
Ursolic acid	7	100	8.0	3,657	3,360	4,793	73
Ursonic acid	12	100	3.9	1,700	2,170	1,485	28
Sodium ursolate	14	100	6.5	1,260	1,400	...	18
Calcium ursolate	20	100	10.0	2,492	2,100	...	35
Methyl ursolate	50	100	53.5	707	885	...	5
Acetyl ursolyl amide	48	110	50.0	890	1,200	2,200	...[c]
Ursolyl amide	48	110	50.0	9,150	8,500	...	197
Ursolyl amine C-28	62	110	31.4	2,220	1,950	...	40
Ursolyl amine C-2	50	110	38.4	7,000	6,500	...	149
Cholic acid	46	110	30.4	9,150	9,800	...	164
Crude ursolic acid	67	110	27.0	5,000	6,300	...	103
Vitamin A	20	100	10.0	1,600	...	...	30
Vitamin E	10	100	3.7	780	...	...	8
Vitamin D_2	16	100	7.0	2,760	...	...	54
Cortisone	20	100	10.0	1,514	...	...	26
Estradiol	11	100	3.7	2,000	...	...	39
Testosterone	13	100	6.0	830	...	...	12

[a] A—End group hydroxyl determination, B—ebullioscopic data, and C—saponification data.
[b] Reported as monomer units per polymer molecule; based on end group hydroxyl determination.
[c] For discussion see results.

TABLE II.—REACTION CONDITIONS AND PROPERTIES OF PROPYLENE OXIDE POLYMERS

Initiator	Reaction Time, Hr.	Reaction Temp., °C.	Polypropylene-Oxide Content, %	Molecular Weight[a] A	Molecular Weight[a] B	Molecular Weight[a] C	Av. Chain Length[b]
Ursolic acid	30	110	8.3	1,015	935	770	10
Ursonic acid	48	135	17.7	1,050	900	727	11
Sodium ursolate	21	135	5.6	835	1,000	...	6
Calcium ursolate	46	135	16.9	1,910	2,200	...	17
Methyl ursolate	72	135	49.0	719	1,000	...	4
Acetyl ursolyl amide	49	135	62.0	1,425	1,400	1,540	16
Ursolyl amide	60	135	45.0	3,882	3,600	...	59
Ursolyl amine C-28	62	135	73.0	2,600	2,200	...	37
Ursolyl amine C-2	72	135	69.0	3,094	2,750	...	53
Cholic acid	46	135	19.5	5,800	5,600	5,010	93
Crude ursolic acid	49	135	29.0	1,184	1,310	...	13

[a] A—End group hydroxyl determination, B—ebullioscopic data, and C—saponification data.
[b] Reported as monomer units per polymer molecule; based on end group hydroxyl determination.

merization reaction similar to the general method was carried out using this compound as the initiator and sodium hydroxide as the catalyst. After only forty-eight hours at 110° (compared to fifty-eight hours for the acetyl ursolyl amide initiated reaction), a polymeric mass was obtained. Attempts were made to isolate pregnenalone acetate from the mixture by Soxhlet extraction using hexane and by preparing the semicarbazone from an aqueous solution; however, these attempts failed. It was concluded, therefore, that pregnenalone acetate had, by some mechanism, become incorporated as a chemical part of the polymer molecule.

It is possible that ethylene oxide reacted with the active hydrogen of the acetyl group or that from the ketonic group. Hydrolysis also might have occurred and left a hydroxyl group in the place of the acetyl group. A mechanism similar to the first is known to take place with the carbanion of ethyl acetoacetate (17).

As noted in Table I, the average molecular weight values, as determined by three methods are not close enough to assign any one structure to the polymer and it is probable that a mixture of materials was obtained. Saponification and hydroxyl value molecular weight are based on a polymer whose initiator contains an intact, unsubstituted acetyl group and two polyoxyethylene chains attached to the amide nitrogen.

The propylene oxide polymer of this same initiator does not show this apparent reaction course. If the acetyl group remains intact and carbanion formation assumes secondary importance, then the only locus of reaction is the sterically hindered amide function. The low yield obtained of this polymer might be explained on this basis. Additional supporting evidence was the failure of pregnenalone acetate initiated reactions to produce any polymeric material with the less reactive propylene oxide.

The propylene oxide polymer of ursolic acid appears to have only one polymer side chain attached to the nucleus. The analogous polymer in the ethylene oxide series has long chains attached to both the carboxyl and hydroxyl groups of ursolic acid, while the propylene oxide polymer seems to contain a hydroxyl chain only. A structure which contains one terminal hydroxyl group is the only one which will correlate the analytical data. A carboxylate polymer would not be justifiable, since the secondary hydroxyl group on the original ursolic acid nucleus would respond to acetylation (as performed in the analysis for hydroxyl value) as would the hydroxyl group of the polymer chain. The steric hindrance imposed on the carboxyl group by the methyl groups in positions 14 and 20, in addition to the more bulky nature of propylene oxide as compared to ethylene oxide, are probably factors which cause hydroxyl reaction to be preferable.

Since the polymers of vitamins A, D_2, and E, and estradiol, cortisone, and testosterone were prepared primarily to see if they were water soluble, only the ethylene oxide series was prepared. All of these polymers were water soluble, vitamin A polymer solutions being opaque. Whether the polymers have retained the pharmacologic activity of the respective initiator is being investigated.

The specific properties of each polymer and reaction conditions are summarized in Tables I and II. Generally, the ethylene oxide polymers were either solids or semisolids, melting from 27° to 52°, and being soluble in water and chloroform and slightly soluble to insoluble in organic solvents such as acetone, alcohol, benzene, hexane, and ether. The propylene oxide polymers were all viscous liquids and were soluble in most organic solvents and insoluble in water.

Polyepichlorhydrin contains a regularly repeating chloromethyl group which may be reacted with magnesium oxide to produce an ether linkage between individual chains (18). If ursolic acid or its derivatives could be used to initiate the polymerization, it too would appear at intervals in this cross-linked polymer. In order to investigate the properties of a polymer whose side chains occupy a comparatively large area with respect to ursolic acid, attempts were made to prepare these compounds. Various heating times, temperatures up to 200°, and sodium hydroxide, aluminum chloride, metallic sodium, and aqueous and concentrated sulfuric acid as catalysts were employed but the desired products did not materialize.

With the same purpose in mind, glycidol was used as the monomer. When this epoxide attaches to an initiator, a secondary alcohol group is generated in addition to the primary hydroxyl group already present. Again, acceptable products were not obtained. It is thought that monomer self-polymerization is a much more predominant reaction than the reaction with ursolic acid.

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Surface Activity of Ethylene Oxide and Propylene Oxide Polymers of Steroidal Compounds*

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The effects of the ethylene oxide polymers of ursolic acid, some of its derivatives, and cholic acid on surface and interfacial tensions and on the formation of oil-in-water emulsions and transparent emulsions are described. In addition, the cotton tape modification of the Draves test was employed for evaluation of these compounds as wetting agents. The propylene oxide polymers of these same initiators were found to be incapable of aiding in the formation of water-in-oil or oil-in-water emulsions. These polymers were evaluated by preparing a series of water-in-oil ointments and by noting their effects on interfacial tension.

IN THE PRECEDING PAPER, the preparation of a series of polymers, designed in structure to fulfill the requirements of surface-active agents, was described. To evaluate these polymers as surfactants, the usual methods were employed using rather widely accepted commercial products as standards of comparison.

EXPERIMENTAL

Surface Tension and Interfacial Tension Measurements.—Perhaps the most common evaluation technique used for any surfactant is to measure its ability to decrease the surface tension exerted by water. One-tenth per cent aqueous solutions of the polymers in the ethylene oxide series were screened for this ability using the du Nouy tensiometer method. After these data were collected, the solutions were overlaid with light liquid petrolatum and the interfacial tensions determined. Since the polymers in the propylene oxide series were all insoluble in water, data were collected for interfacial tensions only. This was accomplished in the same manner as with the ethylene oxide polymers except that the propylene oxide polymers, 0.1%, were dissolved in the light liquid petrolatum, then underlaid with water.

For comparison purposes, data were obtained for 0.1% solutions of Tween 80® (polyoxyethylene sorbitan monooleate)[1] and Aerosol O T[2] (dioctyl sodium sulfosuccinate) along with the ethylene oxide polymers. As the standard of comparison for the propylene oxide polymers, a 0.1% solution of Span 60® (sorbitan monostearate)[1] in light liquid petrolatum was employed. Polyethylene glycol and polypropylene glycol solutions were tested in a similar manner in order to see if the steroidal polymers were more surface active. The results are shown in Table I.

TABLE I.—SURFACE TENSION EFFECTS OF VARIOUS POLYMERS AND CERTAIN STANDARDS[a]

Solution	Surface Tension, Dynes/cm.	Interfacial Tension in Dynes/cm.
Distilled Water	72.0	46.3
Tween 80	42.5	7.8
Aerosol O T	30.8	2.5
Polyethylene Glycol 1540	62.5	31.9
Polyethylene Glycol 4000	62.5	29.9
Ethylene Oxide Polymer of:		
Ursolic Acid	44.1	9.7
Ursonic Acid	42.3	7.8
Sodium Ursolate	43.7	8.6
Calcium Ursolate	42.8	8.2
Methyl Ursolate	43.3	10.6
Acetyl Ursolyl Amide	41.5	9.5
Ursolyl Amide	43.3	9.4
Ursolyl Amine C-28	43.0	12.5
Ursolyl Amine C-2	49.3	17.5
Cholic Acid	52.8	19.4
Crude Ursolic Acid	49.9	18.0
Span 60	...	3.1
Polypropylene Glycol 425	...	25.5
Propylene Oxide Polymer of:		
Ursolic Acid	...	10.3
Ursonic Acid	...	10.5
Sodium Ursolate	...	11.7
Calcium Ursolate	...	14.0
Methyl Ursolate	...	9.8
Acetyl Ursolyl Amide	...	8.5
Ursolyl Amide	...	8.4
Ursolyl Amine C-28	...	9.9
Ursolyl Amine C-2	...	8.0
Cholic Acid	...	8.2
Crude Ursolic Acid	...	10.0

[a] Data presented for solutions of 0.1% solute concentration.

Wetting Agent Evaluation.—The capability of a surfactant to be a good wetting agent is frequently tested by the Draves method. The principle of this test is the comparison of times required for the complete wetting and sinking of a skein of yarn by solutions of the test material and some standard. Since a large number of readings are required for accuracy, various modifications have been suggested. The cotton tape modification of the Draves test was chosen to be used in this work (1). Rather than using skeins of yarn which contain considerable entrapped air, special cotton tapes of uniform specifications were used. Two tape tests are adequate to give accuracy within 5%, while thirteen skein tests are required to give the same degree of accuracy. Since only the ethylene oxide polymers are

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[1] Atlas Powder Company, Wilmington, Del.
[2] American Cyanamide and Chemical Corp., New York, N. Y.

water soluble, this test could not be applied to the propylene oxide polymers. One-tenth per cent solutions of the comparison standards and the various polymers were used and their respective wetting times taken at a temperature of 33°. The results are shown in Table I[7].

Emulsion Tests.—The ethylene oxide polymers, which were designed for oil-in-water emulsification, were used in a series of emulsions and compared with Tween 80. Several emulsion formulations, varying in percentage composition of ingredients, were initially tested. The emulsions containing 40% mineral oil, 5–7% polymer, and 53–55% water were made by mixing in a Waring Blendor for one minute. These emulsions were much too well formed to allow any significant differential comparison.

The formula finally selected for comparison consisted of 70% water, 28% mineral oil, and 2% emulsifier. These ingredients were mixed in a Waring Blendor for ten seconds; the emulsions formed were then passed twice through a hand homogenizer. The emulsions were evaluated immediately after preparation and after two weeks storage at 45°. This storage period is equivalent to approximately six months at room temperature (2). The general appearance, relative viscosity at 31°, approximate particle size as obtained using an ocular micrometer, and globule shapes are recorded in Tables III and IV.

Ointment Tests.—Several attempts were made at preparing 30% oil-in-water and water-in-oil emulsions using 2–4% Span 60, Span 80®,[1] and the propylene oxide polymer of ursolic acid as the emulsifying agents. The ingredients were mixed for one minute in a Waring Blendor, then passed through a hand homogenizer three times. None of these emulsions were good; complete splitting of phases took place within five minutes.

For gross comparative purposes, a water-in-oil ointment formulation was selected. The oil phase portion of the formula consisted of 10% emulsifier, 5% beeswax, 60% petrolatum, and 25% mineral oil (3). This mixture was heated to 65° and 30%

TABLE II.—WETTING TIMES OF ETHYLENE OXIDE POLYMERS AND CERTAIN STANDARDS[a]

Solution	Av. Wetting Time, Sec.
Distilled Water	108.6
Tween 80	22.5
Aerosol O T	1.1
Polyethylene Glycol 4000	42.7
Ethylene Oxide Polymer of:	
Ursolic Acid	33.5
Ursonic Acid	26.5
Sodium Ursolate	19.6
Calcium Ursolate	8.3
Methyl Ursolate	31.3
Acetyl Ursolyl Amide	27.9
Ursolyl Amide	57.0
Ursolyl Amine C-28	54.3
Ursolyl Amine C-2	63.2
Cholic Acid	90.7
Crude Ursolic Acid	57.2

[a] Data presented for solutions of 0.1% solute concentration.

TABLE III.—CHARACTERISTICS OF EMULSIONS MADE USING ETHYLENE OXIDE POLYMERS IMMEDIATELY AFTER PREPARATION[a]

Emulsifier	General Appearance[b]	Relative Viscosity[c]	Approximate Particle Size Distribution %	μ	Globule Shape
Tween 80	Good	1.90	95	1	Round
			5	5	
Ethylene Oxide Polymer of:					
Ursolic Acid	Good	1.81	90	1	Round
			10	10	
Ursonic Acid	Good	2.46	75	1	Round
			25	2.5	
Sodium Ursolate	Good	2.24	95	1	Round
			5	2.5	
Calcium Ursolate	Good	2.28	90	1	Round
			10	5	
Methyl Ursolate	Good	1.77	50	1	Very distorted
			50	35	
Acetyl Ursolyl Amide	Good	3.34	95	1	Round
			5	4	
Ursolyl Amide	Good	2.01	95	1	Round
			5	1.5	
Ursolyl Amine C-28	Good	1.98	95	1	Round
			5	1.5	
Ursolyl Amine C-2	Good	2.73	95	1	Round
			5	2	
Cholic Acid	Good	2.50	90	1	Round
			10	2.5	
Crude Ursolic Acid	Good	3.58	50	1	Slightly distorted
			50	8	

[a] Attempts to make an emulsion using 2% ursolic acid as the emulsifier completely failed, even when the mixing time in the Waring Blendor was increased to one minute.
[b] All of the emulsions seemed to begin creaming within one hour after their preparation. On shaking, uniformity was readily obtained. The emulsions were all white and creamy with the exception of the Tween 80 and the acetyl ursolyl amide polymer emulsions which had a very faint yellow tint.
[c] Determined at 31° using an Ostwald viscosimeter.

TABLE IV.—CHARACTERISTICS OF EMULSIONS MADE USING ETHYLENE OXIDE POLYMERS AFTER TWO WEEKS STORAGE AT 45°

Emulsifier	General Appearance	Relative Viscosity[a]	Approximate Particle Size Distribution %	Approximate Particle Size Distribution μ	Globule Shape
Tween 80	Good	2.08	95	1	Round
			5	5	
Ethylene Oxide Polymer of:					
Ursolic Acid	Some oil-water separation	2.28	85	1	1% distorted
			15	10	
Ursonic Acid	Very slight oil-water separation	1.78	75	1	Round
			25	2.5	
Sodium Ursolate	Some oil-water separation	2.31	95	1	Round
			5	2.5	
Calcium Ursolate	Very good	1.97	90	1	Round
			10	5	
Methyl Ursolate	Poor	2.12	80	2.5	Round
			20	20	
Acetyl Ursolyl Amide	Very poor	1.75	80	1.5	20% distorted
			20	10	
Ursolyl Amide	Poor	1.86	90	1	5% distorted
			10	5	
Ursolyl Amine C-28	Completely split	1.38	80	1.5	1% distorted
			20	10	
Ursolyl Amine C-2	Very slight oil-water separation	2.52	95	1	5% distorted
			5	10	
Cholic Acid	Good	2.55	90	1	Round
			10	2.5	
Crude Ursolic Acid	Very slight oil-water separation	3.43	50	1	Round
			50	10	

[a] Determined at 31° using an Ostwald viscosimeter.

of its weight in water, at 70°, was added with continuous stirring until the ointment was cool. The necessary amounts were chosen in order to prepare two ounces of ointment with each emulsifier. As the comparison standard emulsifier, Arlacel 83® (sorbitan sesquioleate) was used. Some of the ointments bled as much as 60% initially. In these cases, the excess water was poured off and the ointment stored at 45° to see if further bleeding would occur. The general description, bleeding characteristics, and comparative ranks of these ointments, immediately after preparation and after two weeks storage at 45°, are recorded in Table V and Table VI.

Transparent Emulsions.—Three of the polymers prepared in the ethylene oxide series had calculated HLB values (3) which indicated that they might possibly be utilized in the preparation of transparent emulsions. The polymers involved were those initiated by ursolic acid, cholic acid, and crude ursolic acid.

A typical formula consisting of 1% spearmint oil, 15% emulsifier, and 84% water was selected (3). The polymers were dissolved in the oil and water was added slowly with vigorous stirring.

Thin, white, nontransparent emulsions were formed with all three polymers. After twenty-four hours, the emulsions were still well formed but not transparent. These results were not entirely un-

TABLE V.—OINTMENT CHARACTERISTICS IMMEDIATELY AFTER PREPARATION

Emulsifier	Description	Bleeding	Rank
Arlacel 83	Excellent	None	1
Propylene Oxide Polymer of:			
Ursolic Acid	Very poor	50%	6
Ursonic Acid	Very poor	60%	5
Sodium Ursolate	Fair	10%	4
Calcium Ursolate	Poor	20%	5
Methyl Ursolate	Good	Very slight	4
Acetyl Ursolyl Amide	Very good	None	2
Ursolyl Amide	Poor	25%	6
Ursolyl Amine C-28	Very poor	40%	6
Ursolyl Amine C-2	Poor	25%	7
Cholic Acid	Good	Very slight	4
Crude Ursolic Acid	Fair	20%	4
None	Good	Very slight	3

TABLE VI.—OINTMENT CHARACTERISTICS AFTER TWO WEEKS STORAGE AT 45°

Emulsifier	Description	Bleeding	Rank
Arlacel 83	Good	Very slight	2
Propylene Oxide Polymer of:			
Ursolic Acid	Very poor	9%	7
Ursonic Acid	Fair	1%	4
Sodium Ursolate	Fair	1%	4
Calcium Ursolate	Fair	1%	4
Methyl Ursolate	Poor	4%	5
Acetyl Ursolyl Amide	Very good	None	1
Ursolyl Amide	Very poor	8%	6
Ursolyl Amine C-28	Fair	1%	4
Ursolyl Amine C-2	Fair	1%	4
Cholic Acid	Very poor	9%	7
Crude Ursolic Acid	Very poor	9%	7
None	Good	Very slight	3

expected since the HLB values are merely indicative of possibie application.

DISCUSSION OF RESULTS

The ursonic acid-ethylene oxide polymer had the most profound effect on interfacial tension. This tension was 7.8 dynes per centimeter. The lowering of surface tension was in the range of 30 dynes per centimeter with all ethylene oxide polymers. These results are very favorable and comparable to the standards used.

The calcium ursolate-ethylene oxide polymer was, by far, the best wetting agent of the series, although it did not approximate Aerosol O T. It was, however, much better than Tween 80.

On the basis of general appearance, approximate particle size distribution, and storage tests, the calcium ursolate-ethylene oxide polymer also produced the best oil-in-water emulsion. Good emulsions were also obtained using the polymers initiated by ursonic acid, ursolyl amine C-2, cholic acid, and crude ursolic acid.

The ethylene oxide polymers of ursolic acid, cholic acid, and crude ursolic acid had calculated HLB values which indicated that they might be capable of producing transparent emulsions. On preparation of some of these emulsions, this property was found to be lacking. It should be pointed out that these compounds are atypical with respect to the types of substances for which the HLB value calculations were set up.

Of the propylene oxide polymers, those initiated by acetyl ursolyl amide, ursolyl amide, ursolyl amine C-2, and cholic acid were best in the ability to lower interfacial tension. They were not, however, as effective as Span 60 which was the standard of comparison.

In general, the ointments produced using the propylene oxide polymers as the emulsifier were very poor in comparison with the standard. Only one polymer, the acetyl ursolyl amide-propylene oxide polymer, was effective. Initially, the ointment produced by this polymer was slightly inferior to the standard; however, it was more stable and after two weeks storage at 45° was the best ointment in the series.

On the basis of these results, it is believed that some of the ethylene oxide polymers have properties which would make them comparable with other surfactants on the market. If the purification procedure used for ursolic acid could be omitted and the crude product used, commercial production would be simple and inexpensive. Because of the expense involved, it is also important that, if possible, the preparation of derivatives be avoided, even though some of the polymers of these initiators were more effective. Since the calcium ursolate-ethylene oxide polymer, showed the most promise, it might be advisable to attempt the preparation of this initiator salt from crude ursolic acid.

The propylene oxide polymers do not seem to have sufficient merit to warrant further consideration.

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A Polarographic Study of β-Haloethylamine Adrenergic Blocking Agents*

By ROGER MANTSAVINOS† and JOHN E. CHRISTIAN

A polarographic procedure is described for measuring the relative rate of cyclization of the β-haloethyl group of Dibenamine-like compounds to the ethyleneimonium form. This method is based on the rapid reaction of thiosulfate with ethyleneimonium ions. The thiosulfate reaction product of several compounds has been found to be polarographically reducible and with Dibenzyline and Dibenamine the heights of the polarographic waves were found to be proportional to various concentrations of the reducible species. The polarographic waves appear to be irreversible and a two electron reduction has been postulated.

ETHYLENEIMONIUM IONS formed by the cyclization of β-haloethyl groups of Dibenamine-like compounds are believed to be responsible for the adrenergic blocking action of these compounds (1). Although ethyleneimonium ions in solutions of aliphatic β-chloroethylamines (nitrogen mustards) are polarographically reducible (2), ethyleneimonium ions formed by the Dibenamine series of compounds are relatively unstable in solution (3) and do not appear to be reducible at the dropping mercury electrode (D.M.E.). No reducible species could be detected in 60 per cent ethanol solutions of Dibenamine or Dibenzyline buffered with acetate at an apparent pH of 7.0. However, the reaction

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product of several ethyleneimonium intermediates, of the Dibenamine type, with thiosulfate were found to be polarographically reducible. Based on this thiosulfate reaction, polarographic procedures were developed for measuring the relative rate of cyclization of β-haloethylamine adrenergic blocking agents and for determining the amount of thiosulfate reaction product formed. It is of interest to note that although ethyleneimonium forms of Dibenamine-like compounds are not polarographically reducible, Lordi and Christian (4), in a physicochemical study of antithistamines, found ethyleneimonium ions of N-ethyl-N-1-naphthylmethyl-2-chloroethylamine (SY-14) and its thiosulfate reaction product to be polarographically reducible in $M/15$ phosphate buffer, pH 5.9.

EXPERIMENTAL

Materials and Apparatus.—A Sargent Model XXI visible recording polarograph with a D.M.E. and a Lingane-Laitinen H-type electrolysis vessel (5) containing a saturated calomel electrode (S.C.E.) was used for most polarographic work. For kinetic experiments a Carritt type H-cell (6) was used to prevent chloride ion contamination from the salt bridge. Prior to recording polarograms, test solutions were degassed for twenty minutes with tank nitrogen which was purified by passage through a train of vanadous sulfate solution (7) and distilled water. The nitrogen was then saturated with the solvent of the test solution before degassing the test solution. Triton X-100, 0.002% was used as maximum suppressor. The capillary used throughout these studies had a constant $m^{2/3} + {}^{1/6}$ value of 1.853 mg.$^{2/3}$ sec. $^{-1/2}$ in the base solution at -0.25 volt *vs.* S.C.E., under a pressure of 60 cm. of mercury. The base solution, having an apparent pH of 7.0, was made by diluting 20 ml. of an acetate buffer concentrate consisting of 18.8 ml. of 1.0 *N* sodium acetate and 1.2 ml. of 1.0 *N* acetic acid prepared in 60% ethanol. The acetate buffer also served as supporting electrolyte. Analytical reagent grade chemicals were used throughout. Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) was first treated to remove extraneous water (8) before being used to prepare standard stock solutions in 60% ethanol. Experimental samples of β-haloethylamines were generously supplied by the Smith, Kline and French Research Laboratories, Philadelphia. Due to the limited aqueous solubility of these compounds a solvent of 60% ethanol was used in all experiments.

Kinetic Studies.—Whenever thiosulfate is added to a solution containing ethyleneimonium ions, an instantaneous reaction occurs resulting in the formation of an ethyl thiosulfate derivative (3) as illustrated by the following reaction:

$$R_2N\overset{+}{\underset{CH_2}{\underset{|}{\diagup\diagdown}}}CH_2 + X^- + S_2O_3^{=} \rightarrow R_2NCH_2CH_2S_2O_3^- + X^-$$

Since this reaction occurs instantaneously, the rate of thiosulfate consumption becomes an indirect measure of the relative rate of cyclization of a β-haloethylamine into the physiologically active ethyleneimonium ion (3). By polarographically following the change in concentration of thiosulfate with time, the relative rate of cyclization of certain β-haloethylamines was determined. The following procedure was used to measure the rate of cyclization for a number of compounds. A solution containing 20 ml. of acetate buffer concentrate, 1.0 ml. of 0.2% Triton X-100, and a known amount of 1.80×10^{-2} *M* sodium thiosulfate (in excess of the amount of amine to be used) was degassed for thirty minutes in a flask immersed in a water bath maintained at 25° ± 0.5°. A second solution of 60% ethanol was simultaneously degassed under similar conditions. After the solutions were deoxygenated an accurately weighed quantity of β-haloethylamine was dissolved in 50 ml. of degassed ethanol contained in a 100-ml. volumetric flask. To this solution was added the acetate buffer-thiosulfate solution and the resulting solution diluted to 100 ml. with degassed ethanol. The final solution was mixed, and an adequate portion was placed in the solution compartment of a Carritt type H-cell and degassed for a final few seconds. At an arbitrarily chosen zero time, a constant potential of -0.025 volt *vs.* S.C.E. was applied and readings of current were made as a function of time. The impressed voltage corresponded to a point on the plateau of the thiosulfate polarographic wave. When the reaction was essentially complete, as evidenced by a steady current, the chart paper containing the current-time curve was removed and the current readings corrected for residual current before graphically determining the velocity constants. In all cases examined the cyclization process was found to be first order over the time period studied (not over thirty minutes for any given experiment). The accepted velocity constant, k, represents an average of three values of the slopes of curves constructed by plotting the natural log of the limiting current against time. A straight line was drawn through the experimental points by inspection (9). The presence of mercury in the reaction mixture did not influence the progress of the reaction. Table I contains experimental data from which the rate of cyclization of Dibenzyline was calculated. Velocity constants of 0.00928 min.$^{-1}$ for Dibenamine, 0.0144 min.$^{-1}$ for N-(α-naphthylmethyl)-N-ethyl-β-chloroethylamine and 0.00525 min.$^{-1}$ for 1-dibenzylamino-2-chloropropane were obtained when determined by this same method. The above procedure is not applicable to compounds such as certain bromo analogs which cyclize at an extremely rapid rate nor to iodo compounds which interfere with the recording of the thiosulfate wave.

Polarographic Determination of the Thiosulfate Reaction Product.—The ethyleneimonium-thiosulfate reaction product of certain β-haloethylamines, henceforth referred to as ET, gave a well-defined polarographic wave upon reduction at the D.M.E. Since anodic thiosulfate waves are obtained at more positive potentials than cathodic ET waves, both residual thiosulfate and ET can be determined polarographically in the same solution. The following procedure was used for the polarographic determination of the thiosulfate reaction product. An accurately weighed amount of the amine hydro-

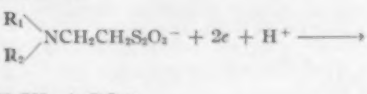
TABLE I.—CYCLIZATION OF DIBENZYLINE IN ETHANOLIC THIOSULFATE SOLUTIONS, 25°

	Initial Concentration of Reactants		
Dibenzyline HCl→	1.00×10^{-3} *M*	1.50×10^{-3} *M*	2.00×10^{-3} *M*
Thiosulfate→	1.80×10^{-3} *M*	2.70×10^{-3} *M*	2.70×10^{-3} *M*
Time (*t*) (Min.)	i_t[a] (Microamp.)	i_t[a] (Microamp.)	i_t[a] (Microamp.)
0	3.84	7.68	5.18
5	3.32	6.72	4.44
10	2.96	5.82	4.00
15	2.76	5.28	3.60
20	2.44	4.89	3.32
	k[b] = 0.0212 min.$^{-1}$	k[b] = 0.0217 min.$^{-1}$	k[b] = 0.0215 min.$^{-1}$
		Mean k value.....	0.02146 min.$^{-1}$

[a] Value of the diffusion current of thiosulfate at time *t*.
[b] *k* values as determined graphically from the slope of a plot of $\ln j_t$ *versus* time.

chloride, sufficient to prepare 100 ml. of a 1.00×10^{-3} *M* to 4.00×10^{-3} *M* solution, was dissolved in 50 ml. of 60% ethanol contained in a 100-ml. volumetric flask. After the amine was solubilized, 20 ml. of acetate buffer concentrate, 1.0 ml. of 0.2% Triton X-100, and a known excess of a fresh stock solution of 2.00×10^{-3} *M* sodium thiosulfate were added to the solution. The resulting solution was diluted to 100 ml. with 60% ethanol, immersed in a constant temperature bath maintained at 25° ± 0.5°, and the reaction allowed to run to completion. The reaction was considered to be complete when a constant diffusion current was obtained by electrolyzing a degassed volume of the test solution from approximately −0.60 to −1.5 volt *vs.* S.C.E. On completion of the reaction, polarograms of degassed test solutions were recorded over this same potential span. A blank solution containing the same volume of thiosulfate as that contained in the standard solution was also run. The amount of thiosulfate consumed was determined by subtracting the diffusion current of the residual thiosulfate in the test solution from the diffusion current of the thiosulfate in the blank solution, and then determining the equivalent concentration from a previously constructed wave height-concentration curve of thiosulfate. This thiosulfate curve was constructed from data obtained under the same conditions at which thiosulfate was made to react with the amine. The amount of thiosulfate consumed in the reaction was taken to be equivalent to the amount of ET in solution. Table II presents experimental data demonstrating the proportionality between the diffusion current obtained by the reduction of ET, and both the concentration of the reducible species and the original concentration of amine. In addition to Dibenzyline and Dibenamine it was of interest to note whether other β-haloethylamines would form reducible ET derivatives, and if the shapes of the reduction waves were suitable for polarographic analysis. The results of such studies are presented in Table III. The experimental data of Table III were obtained by electrolyzing a solution containing an excess of thiosulfate and the hydrochloride of the compound listed. In all cases in which well-defined polarographic waves were obtained the $E_{1/2}$ values were found to shift to more negative potentials with an increase in concentration. The physiologically inactive compound, dibenzylaminoethanol, did not form a reducible thiosulfate substitution product.

Postulated Mechanism of Reduction.—A first approximation of *n* (the number of electrons involved in an electrode reaction) may be obtained from calculations involving the Ilkovic equation. Such calculations, however, presuppose a knowledge of the diffusion coefficient of the substance undergoing electrochemical reaction. Since the diffusion coefficient of the reducible ET species was not known, the value of *n* could not be calculated from the Ilkovic equation. A plausible electrode reaction, derived from a consideration of the electronic nature of the reducible species is indicated by the following equation:



The variation of $E_{1/2}$ values with concentration of

TABLE II.—CALIBRATION DATA FOR DETERMINING ET FORMED IN THE REACTION OF THIOSULFATE WITH DIBENZYLINE AND DIBENAMINE

Dibenzyline·HCl (m*M* Liter^{-1})	Thiosulfate[a] Consumed (m*M* Liter^{-1})	id (ET) (Microamp.)	$E_{1/2}$ *vs.* S.C.E. (Volts)	id[b]/C
		Dibenzyline		
1.00	1.00	3.66	−1.06	3.66
1.50	1.51	5.28	−1.06	3.50
2.00	2.10	7.20	−1.07	3.43
2.50	2.48	9.12	−1.07	3.68
3.00	3.00	11.2	−1.08	3.73
3.50	3.51	13.0	−1.09	3.71
4.00	3.98	14.6	−1.09	3.67
Dibenamine·HCl (m*M* liter^{-1})				
		Dibenamine		
1.00	1.02	4.38	−1.06	4.29
1.50	1.52	6.48	−1.06	4.26
2.00	2.00	8.55	−1.06	4.28
2.50	2.47	10.9	−1.07	4.41
3.00	2.93	13.2	−1.07	4.51
3.50	3.47	15.3	−1.07	4.38
4.00	3.95	17.6	−1.08	4.46

[a] This value was taken to be equivalent to the concentration of ET in solution.
[b] Diffusion current constant of ET.

TABLE III.—CHARACTERISTICS OF CERTAIN ET WAVES

Compound[a] (1.00×10^{-3} *M*)	Description of ET Wave	$E_{1/2}$ *vs.* S.C.E.[b] (Volts)	Description of Residual Thiosulfate Wave
1-Dibenzylamino-2-chloropropane·HCl	Ill-defined (poor plateau)	..	Well-defined
N-(α-Naphthylmethyl)-N-ethyl-β-chloroethylamine·HCl	Well-defined	−0.97	Well-defined
N,N-Dibenzyl-β-bromoethylamine·HCl	Well-defined	−1.05	Well-defined
N-(*o*-Isopropylphenoxyethyl)-N-benzyl-β-bromoethylamine.HBr	Well-defined	-1.04_8	Well-defined
1-Dibenzylamino-2-bromopropane·HBr	Ill-defined	....	Well-defined
N,N-Dibenzyl-β-iodoethylamine·HCl	Well-defined	−1.05	Irregular

[a] Compound used to form the ET derivative by reaction with thiosulfate.
[b] $E_{1/2}$ values for ET waves obtained in solutions containing 1.0×10^{-3} *M* of the amine listed and an excess of thiosulfate.

the reducible species and the shape of the waves indicate that the electrode process is irreversible.

DISCUSSION

These experiments show that polarography, whenever applicable, may be used to measure the relative rate of cyclization of Dibenamine-like compounds as well as the amount of ET formed by the reaction of ethyleneimonium intermediates with thiosulfate. Both of these procedures are based on the rapid thiosulfate reaction.

The relative rate of cyclization of β-haloethyl groups can be measured indirectly by polarographically following the rate at which thiosulfate is consumed by the reaction, providing this reaction is not extremely rapid. The procedure described for kinetic studies is not applicable to β-iodoethylamines since iodide ions produce polarographic waves which interfere with the thiosulfate wave. This interference is probably due to the fact that anodic iodide waves are formed at more negative potentials than chloride or bromide waves (10).

The physiologically inactive compound, dibenzylaminoethanol, did not consume thiosulfate and consequently an ET reduction wave was not obtained. Polarographic data of this kind may serve as an initial screening index of the pharmacological activity of Dibenamine-like compounds, assuming that the ethyleneimonium intermediates are responsible for the pharmacological activity of these compounds.

On the basis of a limited number of experiments no correlation could be drawn between the $E_{1/2}$ values of certain ET derivatives and their pharmacological activity as tabulated by Ullyot and Kerwin in (3).

SUMMARY

1. A polarographic method has been developed for measuring the relative rates of cyclization of certain β-haloethylamine adrenergic blocking agents.

2. The end product resulting from the reaction of some physiologically active Dibenamine-like compounds with thiosulfate was found to give well-defined polarographic waves upon reduction in 60% ethanol, buffered with acetate at an apparent pH of 7.0. The diffusion current obtained by reduction of the thiosulfate substitution products of Dibenamine and Dibenzyline was found to be proportional to various concentrations of the reducible species, as well as the original concentration of the parent β-haloethylamine of the reaction mixture.

3. The physiologically inactive β-haloethylamine, dibenzylaminoethanol, did not consume thiosulfate or form a reducible thiosulfate substitution product.

4. A two electron reduction has been postulated and the ET waves appear to be irreversible.

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The Suitability of Barley as a Host Plant for the Cultivation of Ergot*

By V. E. TYLER, Jr.†

Barley was inoculated with a specific strain of *Claviceps purpurea* using a puncture board and the resultant sclerotia were compared with those obtained from rye plants under similar conditions. As a host, rye proved superior to barley in terms of the percentage of plants infected and quantity and weight of the sclerotia produced. Likewise the alkaloid content of the rye sclerotia was slightly higher than of those obtained from barley. It is concluded that barley is inferior to rye as a host plant for the cultivation of ergot.

RYE, *Secale cereale* L., continues to be recognized as the sole official source of ergot, *Claviceps purpurea* (Fries) Tulasne (1), although the medicinally useful alkaloids may be obtained from sclerotia produced on other plants. This reflects the role of rye as the most common host of the fungus.

The proclivity of rye toward infection in comparison with other cereal grains such as wheat, *Triticum aestivum* L., and barley, *Hordeum vulgare* L., has been reviewed by Guggisberg (2). One of the chief considerations is the fact that under natural conditions infection by conidia is chiefly confined to the flowering period, and rye is largely dependent on cross-fertilization which requires the opening of the glumes and exposure of the gynoecium. Also, it has been established that most of the flowers of wheat and barley are already self-fertilized when the blossoms open, and chance of infection with ergot spores is thereby greatly reduced.

The characteristics of ergot existing as a natural parasite on various members of the *Gramineae* have been the subject of a number of investigations (3–5). Recently, Meinicke (6) has reported on the alkaloid content of ergot produced by the inoculation of various wild grasses with a specific strain of *Claviceps purpurea*. The results of his experiments indicate that the host may influence the alkaloid content of the parasite in a quantitative manner only. It is apparent, therefore, that quantitative reports of the alkaloid content of ergot sclerotia formed on different grasses are not comparable, one with another, unless it is known that both hosts were infected with the identical strain of the fungus.

With the advent of infection methods which are not dependent upon the periodic opening of the blossoms of the various plants (7) and the availability of specific ergot strains obtained from single-spore cultures, it appeared of interest to compare the quantity and quality of ergot produced by puncture-inoculation of rye and another host plant under experimental conditions.

The availability of a variety of barley, producing plants about one-third of which were male sterile and therefore quite susceptible to infection on theoretical grounds, accounted for its selection as the plant to be compared with rye under field conditions with respect to its relative suitability as a host for ergot.

EXPERIMENTAL

Inoculation.—Winter rye, *Secale cereale* var. *caribou* and spring barley, *Hordeum vulgare*, heterozygous for male sterility, were planted in rows 10 feet long, spaced 12 inches apart. The rows selected for inoculation were part of a series of larger plots employed for general agronomic research and received no special treatment. Although widely separated, the two plots were quite similar in terms of soil, drainage, exposure, and related factors. In order to obtain samples large enough to be significant, plots containing approximately 1,000 spikes of each grain were treated.

The inoculum was prepared by inoculating 1 inch × 8 inch culture tubes, containing slants of a casein hydrolysate-mannitol-basic nutrient medium (8) solidified with 2% agar, with an ergotamine-producing strain[1] of *C. purpurea* originally selected from a mono-conidiaspore isolate. These cultures were prepared four to six weeks prior to need and stored at 25°, during which time a profuse mycelial development took place. The mycelial mat was then removed from 4–6 slants, depending upon the quantity of growth present, homogenized in a Waring Blendor with a small quantity of distilled water and this slurry diluted to a volume of 480 ml. This constituted the finished inoculum which was applied to the plants with a puncture board as described by Guggisberg (9). The puncture board employed in this investigation measured approximately 12 × 13 cm. and was set with eyed needles which projected

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[1] Supplied through the courtesy of Dr. N. Bekesy, Budapest, Hungary.

5–7 mm. from its surface. The needles were evenly spaced and averaged seven per sq. cm. of board surface.

By May 24, 1957, the winter rye had matured sufficiently so that most of the plants were in the developmental stages labeled I, II, and III by Bekesy (10). Inoculation was carried out late in the afternoon so that the spores might receive the benefit of the cool night hours during their initial germination stages. The spring barley was considered ready, and consequently inoculated, on June 10, the majority of plants having reached stages I and II by this date. The fact that barley is known to undergo self-fertilization before emergence of the spike from the sheath accounted for the inoculation of this plant at a somewhat earlier stage of development.

No unusual weather conditions were noted subsequent to the inoculations and the fungus appeared to develop normally on both hosts, honeydew being evident after six or seven days. It has been established by Loo and Lewis (11) that ergot sclerotia reach a maximum alkaloid content after nineteen days of development although they are still increasing in weight at this time. Therefore, in this experiment a growing period of one month was arbitrarily selected as being sufficient time to allow most sclerotia to mature, at least to maximum alkaloid content, but not long enough to allow appreciable losses to occur by quantities of large ripe sclerotia falling from the drying spikes of the plants. At this time all of the spikes in each of the experimental plots were harvested, regardless of infection, and subjected to analysis.

Analytical Procedures.—Total spikes were counted as were the number infected with the fungus. The sclerotia were then separated, the maximum number in a single spike being noted during the process. All sclerotia were counted, the entire sample weighed and its moisture content determined by a conductometric method (12). Samples of each type of ergot were then ground to a No. 20 powder in a Wiley Laboratory Mill and fat determinations carried out on duplicate 2-Gm. samples as described by Jones and Tyler (13). The defatted samples were next milled to a No. 60 powder and duplicate samples assayed for water-soluble and water-insoluble alkaloids by the procedure of Silber and Schulze (14). Water-soluble alkaloids were calculated as ergonovine and reported on the basis of a moisture-free, fat-free sample. The water-insoluble alkaloids were calculated as ergotoxine and reported on the same basis. Portions of the water-insoluble extracts not used for assay were made alkaline, extracted with ether and these ethereal extracts chromatographed by the method of Meinicke (6) to determine the composition of the water-insoluble alkaloid fraction. The water-soluble fractions treated in the same manner were chromatographed in a butanol-acetic acid-water (4:1:5) system as described by Tyler and Schwarting (15) to determine their composition. The results of these analyses are recorded in Table I.

TABLE I.—COMPARISON OF THE ERGOTS PRODUCED ON RYE AND BARLEY

	Rye	Barley
Total no. of spikes	1,115	989
No. of infected spikes	722	211
Infected spikes (%)	64.8	21.3
No. of sclerotia	2,371	1,159
Av. no. sclerotia per infected spike	3.3	5.5
Max. no. sclerotia per infected spike	22	28
Fresh wt. sclerotia (Gm.)	57.27	22.44
Av. wt. of single sclerotium (mg.)	24.1	19.4
Av. wt. of ergot per infected spike (mg.)	79.3	106.4
Moisture (%)	6.7	7.4
Fat (%)	15.6	10.23
Alkaloid—water-sol. (%)	0.045	0.027
Alkaloid—water-insol. (%)	0.631	0.515
Alkaloid identity—water sol.	Ergonovine	
Alkaloid identity—water-insol.	Ergotamine (90% of complex) Ergosine (little) Ergotamine (little) Ergocristine — Ergocornine (faint trace) Ergokryptine (trace)	

DISCUSSION

These data indicate that rye is superior to barley as an ergot host plant in terms of quantity and weight of sclerotia formed in the total plots. Nearly two-thirds of the rye spikes treated developed one or more sclerotia in comparison to slightly more than one-fifth of the barley spikes, and the yield from the entire rye plot was more than double that of the barley on a weight basis. The individual rye sclerotia were slightly larger and contained a slightly higher alkaloid content than those of barley although the qualitative composition of the alkaloidal mixture of both ergots was identical. On the other hand, the barley plants which did become infected produced more sclerotia (av. 5.5) per spike than the rye (av. 3.3). This seems to indicate that the difference in these two plants as hosts for ergot is due to the greater susceptibility of the rye to infection, even when both are infected by an injection technique which circumvents the effects produced by differences in flowering habits. Apparently this greater susceptibility applies to the rye plants as a group but not necessarily to the individual since a single, infected barley plant yielded, on the average, more ergot sclerotia both in number and total weight than a single infected rye plant; however, fewer barley plants in the group became infected.

The reasons for these differences in susceptibility of the two plants are obscure although the sterile character of the variety of barley employed was probably not without influence, particularly on the number of sclerotia which developed on the individual barley spikes. Garay (16) has investigated some of the factors which affect the germination of ergot conidia with reference to the host plant. Hydrogen ion concentration and osmotic pressure were shown to have marked effects on germination, but no stimulation could be shown to result following treatment with extracts of rye tissues. The same extracts, after autoclaving, did stimulate growth of saprophytic cultures of ergot, thus confirming the report of Berman and Youngken (17). The impor-

tance of these various factors with reference to this experiment is merely speculative, but they may be used as a starting point for further study on the suitability of various cereals as hosts for the cultivation of ergot.

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A Quantitative Papergram Assay Method for Prednisolone in Tablet Formulations*

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The quantitative assay of prednisolone in tablet formulations herein described involves separation of the steroid from the nonsteroidal tablet components by paper chromatography. Elution of the steroid spot from the papergram with methanol is followed by measurement of the ultraviolet absorption of the eluate at 224 mμ, 244 mμ, and 260 mμ. The absorbance values measured at these three wavelengths are used to calculate the true steroid concentration. The computation provides correction for the small but significant amounts of ultraviolet absorbing materials which are invariably present in the papergram eluates.

THE NEED for an assay method specific for prednisolone became apparent when tablet formulations containing prednisolone, acetylsalicylic acid, and an antacid were subjected to an accelerated stability study at 70°. In this study, assays by the conventional triphenyltetrazolium assay showed that, after an initial decrease, the prednisolone concentration appeared to increase upon prolonged exposure to 70° (1). This apparent increase indicated the formation of a reducing substance in the dosage form during the course of the heat stress.

The principle of the procedure described in this paper evolved from the methodology previously developed in our laboratories for the quantitative assay of progesterone in the presence of 11α-hydroxyprogesterone (2). The present method involves the paper chromatographic separation of prednisolone from major undesirable components followed by spectrophotometric measurements at three different wavelengths to compensate for minor amounts of nonsteroidal absorbing substances in the eluates.

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EXPERIMENTAL

Reagents and Material

Reagents.—Chloroform, A. R. (Mallinckrodt Chemical Works); absolute ethyl alcohol, (Commercial Solvents Corporation); absolute methyl alcohol, A. R. (Mallinckrodt Chemical Works); propylene glycol, U. S. P. (Carbide and Carbon Chemicals Corp.); ethylene dichloride, 99%, (Dow Chemical Company); toluene, A. R. (Mallinckrodt Chemical Works); acetic acid, C. P. (E. I. DuPont de Nemours & Co.); prednisolone: Delta-Cortef® (Upjohn brand of prednisolone) standard solution: 1 mg./cc. in 95% ethanol[1]; and chromatographic paper, Eaton Dikeman 613, 6 inches × 23 inches.

Apparatus.—Semimicro evaporator constructed in these laboratories; spectrophotometers—a Beckman Model DU and a Cary Recording spectrophotometer Model 14 were employed; pipet—a 0.2-cc. pipet graduated in 0.01 cc. (MISCO); and papergram scanner (3).

Development of the Papergram Assay Procedure

Previous experience has shown that the development of a quantitative paper chromatographic assay consists of the following steps:

Selection of a suitable solvent system.—The solvent system of choice, besides giving as complete as

[1] Dr. T. Chulski of these laboratories showed this material to be 99.82% pure by solubility analysis.

possible separation of the steroid from nonsteroidal components, should not excessively contribute to the ultraviolet absorption of the papergram eluates.

Determination of nonspecific ultraviolet absorption.—Small contributions to the ultraviolet absorption of the papergram eluates were expected to result from the elution of the paper and the solvents (2) as well as from the elution of minor tablet ingredients. Therefore, an accurate determination of the background absorption was necessary to provide for its mathematical compensation.

Determination of steroid recovery.—The average recovery of known amounts of steroid has to be determined for each solvent system.

Calculation of the true steroid concentration.—A mathematical formula has to be developed which compensates for the impurities found to be present in the papergram eluates.

Selection of a Papergram Solvent System.—A tablet formulation containing prednisolone, acetylsalicylic acid, calcium carbonate, sucrose, lactose, starch, ethylcellulose, talc, and mineral oil was exposed to 70° temperature for seventy-four hours. The neutral chloroform extract of the crushed tablets was essentially free of prednisolone as indicated by the ultraviolet and infrared spectra of the extract. This extract, however, had considerable absorbance when assayed with triphenyltetrazolium (4).

Of all the solvent systems investigated the propylene glycol-toluene-methanol system of Zaffaroni as modified by Reineke (2) with acetic acid (PTFA-system) gave the best separation of mixtures of prednisolone (32 γ), of acetylsalicylic acid (50 γ) and of the above described extract (50–100 γ). The mobile phase consists of 2.0% glacial acetic acid added to toluene previously saturated with propylene glycol. The stationary phase is propylene glycol-methanol in a 1:1 ratio.

Quantitative Determination of Nonsteroidal Background Absorption Resulting from the Elution of Papergram Blanks.—Two empty sheets properly pretreated with the stationary phase were developed with the mobile phase for thirty-nine hours. After a drying period of two hours at 108°, two parallel strips 6 cm. wide (beginning 5 cm. below the origin and extending 25 cm. downwards) were cut out. These two strips were subdivided into smaller pieces of approximately 6 × 3.5 cm. size. These latter pieces were then cut into bits of 1 cm.² size and were eluted in individual 250-cc. Erlenmeyer flasks with 50 cc. of dry methanol. The ultraviolet absorption of the eluates was determined with the Cary spectrophotometer. The results are given in Table I.

Quantitative Determination of the Background Absorption Resulting from Nonsteroidal Formulation Components.—Forty hours of development on the PTFA system gave excellent separation of prednisolone and acetylsalicylic acid. To determine the degree of interference from minor amounts of U. V. absorbing compounds six tablet formulations were prepared which contained all the ingredients except prednisolone, but which differed from each other with respect to the carbohydrate component. After storage at 70° these tablets were extracted with chloroform.

Approximately equal aliquots of the extraction residues in chloroform solution were applied to paper-strips and the strips were developed and processed as described in the final assay method. Areas (approximately 6 × 3.5 cm.) were cut from the dried sheets which corresponded in size and position to the size and position of the areas occupied by the prednisolone spots in the assay procedure. Each area was then cut into small bits of 1 cm.² size and eluted with 30 cc. of methanol.

The ultraviolet absorption of the eluates was determined with the Cary spectrophotometer. Visual inspection of the curves indicated that all of them had a similar shape with one of the maxima being at 260 mμ. The absorbances at 224 mμ, 244 mμ, and 260 mμ are given in Table II.

TABLE I.—BACKGROUND ABSORPTION RESULTING FROM THE ELUTION OF BLANK PAPERGRAMS

Sample	A 224 mμ	A 244 mμ	A 260 mμ
A1	0.042	0.015	0.019
A3	0.020	0.002	0.004
A5	0.010	0.002	0.006
A7	0.048	0.015	0.020
B1	0.012	0.002	0.005
B2	0.018	0.003	0.007
B4	0.020	0.004	0.007
B6	0.040	0.012	0.014
B8	0.046	0.020	0.020

Average Ratios: A 224 mμ/A 244 mμ = 3.41; A 260 mμ/A 244 mμ = 1.36

TABLE II.—BACKGROUND ABSORPTION RESULTING FROM PAPERGRAM ELUTION OF NONSTEROIDAL TABLET COMPONENTS

Carbohydrate	Time Exposure to 70°, Hr.	Aliquot of Extract Applied to Paper, mg.	Absorbance of Papergram Eluates[a] A 224 mμ	A 244 mμ	A 260 mμ
Starch	52	0.982	0.090	0.042	0.039
Lactose	52	0.970	0.029	0.022	0.014
Methylcellulose	52	0.994	0.020	0.014	0.012
Sucrose	52	1.015	0.013	0.008	0.007
D-Sorbitol	52	0.959	0.030	0.020	0.013
Dextrose	52	0.975	0.005	0.004	0.002
Dextrose	76	1.003	0.023	0.016	0.015

Average Ratios: A 224 mμ/A 260 mμ = 2.04; A 244 mμ/A 260 mμ = 1.17.

[a] These values have been corrected for the average blank absorption (cf. Table I) at each wavelength.

Determination of Steroid Recovery.—Two aliquots of a methanolic solution containing 4.00 mg. of prednisolone were applied to the paper in the manner described under the final assay method. After forty hours development in the PTFA-system, each prednisolone spot was eluted from the paper with 30 ml. of methanol. The prednisolone concentrations were determined at 244 mμ and the values corrected for the average background absorption eluted from the paper (cf. Table I). Two sets of experiments each consisting of six determinations gave an average recovery of 95.1% which is in good agreement with the previously reported recovery of progesterone (2).

Calculation of Prednisolone Concentration in Papergram Eluates.—*Formula I.*—While the papergram eluates contained three ultraviolet absorbing components (x, y, and z, below), only the concen-

tration of the steroid was of interest. This required absorbance readings at three wavelengths and the use of a general three-component equation for the desired steroid component, x, in the three-component system according to known procedures (5). The equation for the steroid concentration was thus:

$$C_x = \frac{A_1(a_{2y}a_{3z} - a_{3y}a_{2z}) + A_2(a_{3y}a_{1z} - a_{1y}a_{3z}) + A_3(a_{1y}a_{2z} - a_{2y}a_{1z})}{a_{1x}a_{2y}a_{3z} - a_{1x}a_{3y}a_{2z} - a_{2x}a_{1y}a_{3z} + a_{2x}a_{3y}a_{1z} + a_{3x}a_{1y}a_{2z} - a_{3x}a_{2y}a_{1z}}$$

Where: x = absorption due to prednisolone; y = absorption of the paper blanks; z = absorption contributed by nonsteroid tablet components; a = absorptivity; A = absorbance; C = concentration in Gm./liter; and 1, 2, 3, = 224, 244, 260 mμ, respectively.

Since in the present problem the absolute quantities of the components y and z are of no interest the ratio values derived from Tables I and II are substituted for the corresponding a-values. The reference data for prednisolone in methanol are:

$a_{224\ m\mu} = 24.55$; $a_{244\ m\mu} = 41.12$; $a_{260\ m\mu} = 28.79$.

With these values the following equation was obtained:

$$C_x = \frac{1.95A_3 - 0.64A_1 - 0.59A_2}{15.48}$$

where C_x = concentration of prednisolone in Gm./L.

Incorporation into the equation of the 95.1% recovery of the steroid from the papergram gave the final form of the equation:

$$C_{\text{prednisolone}} = \frac{1.95A_3 - 0.64A_1 - 0.59A_2}{14.72} \quad \text{(Eq. 1)}$$

This formula was applied to the determination of prednisolone in stability samples of a large number of tablet formulations. The results showed conclusively that in many instances the triphenyltetrazolium assay gave higher potencies of prednisolone than the papergram assay. The results of the papergram assay, therefore, were used to screen for the more stable formulations.

Formula II.—This screening process showed that a double compressed tablet possessed excellent stability properties. It was considered necessary to determine the background absorption of this specific formulation to increase the accuracy of the method for the assay of the stability samples. Steroid free tablets were, therefore, made and subjected to 70° for 24, 48, and 72 hours. Extractions of the tablet cores, paperchromatography of the extracts, and determination of the ultraviolet absorption of the papergram eluates gave the values listed in Table III.

When these ratios and the 95.1% recovery value were incorporated into the above mentioned general equation the following formula was obtained:

$$C_{\text{prednisolone}} = \frac{0.07A_2 + 1.05A_3 - 0.44A_1}{21.36} \quad \text{(Eq. 2)}$$

This formula was used for the results discussed and analyzed in the subsequent paper (6).

Final Assay Method

Tablet Extraction.—Ten tablets were taken as the sample for analysis to reduce sampling error. The cores containing the steroid were separated from the outer coat, weighed, and finely powdered. Three aliquots were weighed with each aliquot corresponding to three tablets. One of these aliquots was used for the papergram assay and two for the two tetrazolium assay procedures "B" and "C" (6).

The aliquot was slurried in 10 cc. of deionized water and 2.5 cc. of a 25% sodium carbonate solution. The slurry was extracted with 50, 30, and 30 ml. of chloroform, respectively. The extracts were evaporated in the semimicro evaporator. The residue was transferred into 1-cc. volumetric flasks with small amounts (approximately 1 cc.) of a 1:1 mixture of chloroform-ethylene dichloride. Each transfer volume was evaporated by passing a stream of purified nitrogen through the volumetric flask. The final volume was adjusted to 1 cc.

Paper chromatography.—Duplicate spots each made from 0.1 cc. of the same sample were applied to the "origin" of one paper sheet. Each spot occupied a site 4 cm. in length which was so spaced that 2-cm. and 3-cm. blank spaces remained between each spot and the long edge of the paper, and between the two spots, respectively. The descending papergram was developed in an air-tight battery jar. After approximately forty hours of development, the sheets were removed from the development chamber, dried in a hot air oven for two hours at 105°, and processed as described in detail by Reineke (2).

An aliquot of the eluate was read in the Beckman DU spectrophotometer.

The absorbance values which were obtained from the duplicate analysis of an authentic sample are given in Table IV.

TABLE III.—NONSTEROIDAL BACKGROUND ABSORPTION RESULTING FROM PAPERGRAM ELUTION OF A SPECIFIC TABLET FORMULATION

Sample	Time Exposed at 70°	A 224 mμ	A 244 mμ	A 260 mμ
1A	24	0.034	0.011	0.010
1B	24	0.044	0.017	0.017
2A	48	0.069	0.032	0.029
2B	48	0.045	0.018	0.017
3A	74	0.055	0.022	0.020
3B	74	0.035	0.017	0.017

Average Ratios: A 224 mμ/A 260 mμ = 2.56; A 244 mμ/A 260 mμ = 1.06

These values were then inserted into equation 2, thus:

$$C_{\text{prednisolone}} = \frac{0.07 \times 0.642 + 1.05 \times 0.452 - 0.44 \times 0.408}{21.36} = \frac{0.3400}{21.36} = 1.59 \times 10^{-2}\ \text{Gm./L.}$$

The concentration of prednisolone for one tablet was calculated from the above data in the following manner: Since an aliquot a (0.1) of the extractives from b (3) tablets was applied to the papergram and

TABLE IV.—ABSORBANCE VALUES FROM PAPERGRAM ELUATES OF AN AUTHENTIC SAMPLE

Sample Aliquot	A_1 (224 mμ)	A_2 (244 mμ)	A_3 (260 mμ)
2A	0.408	0.642	0.452
2B	0.398	0.623	0.442

the steroid spot was eluted with d (0.03) liters of solvent, the calculation became:

$$C_{\text{prednisolone/tablet}} = \frac{C_{\text{prednisolone}} \times d}{a \times b} \text{ Gm.} =$$

$$\frac{1.59 \times 10^{-2} \text{ Gm./L.} \times 3 \times 10^{-2} \text{ L.}}{10^{-1} \times 3} \text{ or}$$

$$C_{\text{prednisolone/tablet}} = 1.59 \times 10^{-3} \text{ Gm.}$$

Table V gives a comparison of values which were obtained by different procedures of calculation from the absorbances listed in Table IV.

TABLE V.—CALCULATION OF PREDNISOLONE CONCENTRATION (MG./TABLET) FROM ABSORBANCES OF TABLE IV BY DIFFERENT PROCEDURES

Sample Aliquot	Method of Calculation		
	A[a]	B[b]	C[c]
2A	1.59	1.56	1.47
2B	1.56	1.51	1.42

[a] Calculated according to Formula II.
[b] Calculated from A_2 (244 mμ) alone without consideration of any correction for background absorption or of 95.1% recovery from the paper.
[c] Calculated from A_2 (244 mμ) alone after correction of A_2 for background absorption eluted from the paper and with consideration of 95.1% recovery of steroid from papergram.

Table V shows that calculation of the steroid concentration by formula II gives the highest values. That these values are very close to the true concentration was determined by processing two accurately weighed samples of prednisolone through the whole analytical procedure. The final results were 2% and 3% low, respectively.

DISCUSSION

A reproducible paperchromatographic assay has been developed which is highly specific for prednisolone in tablet formulations. Although the assay is rather tedious it is superior to conventional methods of 17-hydroxycorticosteroid assay because it almost completely eliminates tablet components and degradation products which might interfere with other methods. The method is designed in such a way that it compensates for minor interferences which are still present after prolonged paper chromatographic separation. Therefore, it is ideal for establishing the veracity of other, less tedious methods.

The paper chromatographic method can also be used for the qualitative determination and characterization of possible artifact material. This may be helpful in uncovering and interpreting imcompatibilities between components in the formulation. To do this, the development of the papergram is interrupted before the artifact spot disappears from the end of the sheet. For example, an inferior prednisolone-acetylsalicylic acid formulation was subjected to the paper chromatographic assay procedure. An artifact area was eluted from the papergram which was subsequently identified by micro-infrared spectroscopy as prednisolone acetate (7). This indicated an undesirable transesterification between prednisolone and acetylsalicylic acid. Knowing the nature of these possible interactions facilitates the formulation of a stable product.

This paper chromatograpnic assay should be applicable, with minor modifications, to the determination of prednisolone in many other dosage forms.

SUMMARY

1. A quantitative paper chromatographic assay for prednisolone in tablet formulations has been developed and applied to the development of a stable prednisolone formulation containing acetylsalicylic acid.

2. Because of its specificity this assay is superior to the triphenyltetrazolium assay in the presence of tablet ingredients which interfere with the latter method.

3. The assay is not recommended for the routine analysis of tablet formulations with good stability. Its application is advantageous, however, if the reliability of routine assay such as the triphenyltetrazolium method has to be established.

4. The versatility of the paper chromatographic assay is discussed in relation to the problem of qualitative interpretation of imcompatibilities.

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Comparison of Methods of Assay of Prednisolone in Compressed Tablets*

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A paper chromatographic method and a method based on chloroform extraction followed by a tetrazolium assay are shown to give similar results in the determination of prednisolone in certain compressed tablets after storage at room temperature and at elevated temperatures. A method based on alcoholic extraction of the steroid followed by a tetrazolium assay is shown to give higher values of potency than the other two methods on most of the stability samples. The increased stability of prednisolone in compressed tablets when the tablets are stored with a desiccant is noted.

THIS COMMUNICATION is concerned with a comparison of three methods of assay of prednisolone in compressed tablets. Method "A" is the paper chromatographic procedure of Meister, *et al.* (1), which without modification can be used for the assay of tablets containing both reducing sugars and acetylsalicylic acid. Method "B" is a chloroform extraction-tetrazolium method which can be used to assay tablets containing reducing sugars and can be easily modified to assay tablets also containing acetylsalicylic acid. Method "C" is an alcohol extraction-tetrazolium method which is unsatisfactory when a reducing sugar such as lactose is present or when applied to certain aged prednisolone tablets.

Previously described colorimetric methods for the determination of 17-hydroxycorticosteroids involve the reduction of a tetrazolium salt in alkaline solution by the ketol group of these steroids (2–8).

Mader and Buck (2) applied the tetrazolium color assay to various steroids and reported that cortisone acetate could be separated from reducing sugars such as lactose by extraction of the steroid with absolute ethyl alcohol or isopropyl alcohol. We have found that the solubility of lactose in absolute ethyl alcohol is sufficient to cause considerable interference in the tetrazolium assay of prednisolone.

The chemical reduction of 2,3,5-triphenyltetrazolium chloride (tetrazolium salt) in alcoholic solution by prednisolone in the presence of approximately 0.009 *N* tetramethylammonium hydroxide is rapid and essentially complete after thirty to thirty-five minutes. Providing only prednisolone is present the absorbance of the colored solutions at the wavelength of maximum absorption (485 mμ) remains essentially constant over the range of thirty-five to sixty minutes. An increase in absorbance with time, in the range of thirty-five to sixty minutes, indicated that an interfering nonsteroidal substance is present. Izzo, Burton, and Keutmann (7) reported similar data for other steroids possessing an alpha ketol side chain. The values of prednisolone potency reported below were calculated from the plateau values of absorbance, i. e., from the absorbance values of the sample and standard after the level portion of the absorbance *versus* time curve had been reached.

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EXPERIMENTAL

Reagents.—*Tetrazolium Solution.*—A 0.5% w/v solution of 2,3,5-triphenyltetrazolium chloride (Dajac Laboratories) in absolute alcohol.

Base Solution.—One milliliter of a 10% aqueous solution of tetramethylammonium hydroxide (Eastman Organic Chemicals No. 1515) was diluted with 9 ml. of absolute alcohol. The solution was filtered through Whatman No. 1 filter paper.

Standard Prednisolone Solution.—A solution containing 10 μg. of specially purified prednisolone per ml. of absolute alcohol. Prednisolone, absolute ethyl alcohol, and chloroform were the same as described by Meister, *et al.* (1).

Samples.—Prednisolone tablets, containing no lactose nor acetylsalicylic acid, were prepared from the same formula but by two different processes. Those prepared by one process are designated Lots I and IA and those prepared by the other process are designated Lots II and IIA. The results reported in Table I led to the following sampling technique. Three aliquots, each representing three 1.5-mg. tablets, were taken from 10 finely powdered tablets. One of the aliquots was assayed by method "A," one by method "B," and one by method "C." Assay values plotted in Figs. 1 through 4 and tabulated in Table II resulted from this type of sampling.

Assay Methods.—*Method "A"* was the paper chromatographic method reported by Meister, *et al.* (1).

Method "B."—The aliquot of powder was mixed with 4 ml. of deionized water in a 500-ml. volumetric flask. About thirty minutes later approximately 250 ml. of chloroform was added, the flask was stoppered, shaken for about two minutes, then allowed to stand for fifteen to thirty minutes. Sufficient chloroform was added to bring the volume to the bottom of the neck, the flask was stoppered and

shaken again for about two minutes. Chloroform was added to the fiducial mark of the flask. The flask was stoppered, shaken, and allowed to stand until the layers separated. After removing the water layer above the fiducial mark with a vacuum hose two 20-ml. aliquots were withdrawn from the center of the flask. Each aliquot was transferred to a 25-ml. glass-stoppered flask containing three glass beads. Most of the chloroform was evaporated on a steam bath but the last traces were removed by suction. The steroid residue was dissolved in 20 ml. of absolute ethyl alcohol, 2.0 ml. of tetrazolium solution were added followed by 2.0 ml. of base solution. The contents of the flask were mixed by swirling. After thirty-five and forty-five minutes the absorbance was determined at 485 mμ on a Beckman spectrophotometer, Model DU. A standard solution of prednisolone and a blank consisting of absolute alcohol were carried through the identical procedure.

Method "C."—The aliquot of powder was transferred to a 500-ml. volumetric flask with the aid of 250-ml. of absolute alcohol. The flask was stoppered and shaken in a shaking machine for two and one-half hours which was shown to be sufficient for complete extraction of the steroid. The flask was then made up to volume with absolute alcohol and the contents mixed by inversion. An aliquot of about 30 ml. of the extract was centrifuged at 20,000 r. p. m. until the supernatant was optically clear. The remainder of the procedure was identical to that described for method "B" except that one-half the volumes of alcoholic solution and reagents and a Beckman spectrophotometer, Model B were used.

For methods "B" and "C" potency of the tablets was calculated by means of the equation:

$$\text{Milligrams of prednisolone per tablet} = 5/3 \times (A_{\text{sample}}/A_{\text{standard}})$$

where A_{sample} is the absorbance of the sample at 485 mμ in the plateau region and A_{standard} is the absorbance of the standard at the same wavelength and after the same time.

RESULTS AND DISCUSSION

Freshly Made Tablets.—The three methods of assay gave the same average potency of prednisolone in freshly made tablets. Table I shows the results obtained with Lot I by methods "B" and "C" and with Lot IA by all three methods. The average of the two series of assays obtained by method "C" on Lot I were tested against the average obtained by method "B" on Lot I by Student's t-test (9*a*). Similarly the averages of the two series of assays obtained by method "C" on Lot IA were tested against the averages obtained by method "B" on Lot IA. In both cases the t-values were less than unity and no significant difference between averages by the two methods could be shown. Method "A" gave identical results in the case of Lot IA, the range being 1.53 ± 0.01 whereas the 95% confidence interval about the mean for method "B" was 1.52 ± 0.03 and for method "C" was 1.53 ± 0.02.

The repetitive assays on groups of three tablets of Lots I and IA by methods "B" and "C" disclosed that the distribution of prednisolone per tablet in Lot IA was much more uniform than in Lot I. This was confirmed by comparing the standard deviations associated with samples (10) for Lots I and IA. To reduce this sampling error in subsequent assays the sampling procedure discussed under "Samples" was used in all subsequent investigations. Duplicate assays were run on each extract by each method in order to estimate precision.

Short Term Stability Tests Carried Out at 70°.—In order to quickly screen formulas, methods of preparation, and packaging variations, samples of the tablets were stored at 70° in an air-circulating oven equipped with an automatic temperature recorder for varying periods up to seventy-two hours.

A blank tablet prepared with the same formula and method of preparation as Lots I and IA but without prednisolone was stored at 70° in sealed bottles with metal-liner caps without Humi-Caps[1] for twenty-four, forty-eight, and seventy-two hours. The samples were analyzed by methods "A" and "C." Results of analysis of these samples by method "A" were incorporated in the three-component equation used to calculate potency on the other samples (1). By method "C" the twenty-four and forty-eight hour samples of these tablets gave a "potency" equivalent to 0.03 mg. of "prednisolone" per tablet and the seventy-two hour sample gave a "potency" equivalent to 0.08 mg. of "prednisolone" per tablet.

Tablet lot IA, stored at 70° with Humi-Caps in the bottles, when assayed by method "A" and "B" exhibited a loss of 9.2% of the initial prednisolone content (1.53 mg./tablet) during the first twenty-four hours but no more loss in potency during the next forty-eight hours. Results obtained with method "C" on the same samples were all much higher, the mean value being 1.56 mg. of prednisolone per tablet in the twenty-four to seventy-two hour range. These results are plotted in Fig. 1.

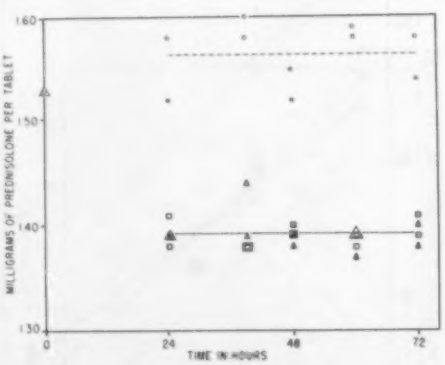

Fig. 1—Plot of milligrams of prednisolone per tablet against time in hours for lot IA stored at 70° in sealed bottles with metal-liner caps and Humi-Caps. △—Method "A"; ⊡—method "B"; ⊙—method "C."

[1] Humi-Caps—A plastic container enclosing approximately 0.75 Gm. of dried silica gel manufactured by Dri Aire Incorporated, South Norwalk, Conn.

TABLE I.—COMPARISON OF ASSAY RESULTS OBTAINED BY THREE DIFFERENT METHODS AND INVESTIGATORS ON DIFFERENT SAMPLES OF THE SAME FRESHLY MADE TABLETS

Potency: Milligrams of Prednisolone Per Tablet							
Lot I[a]			Lot IA[a]			Lot IA[b]	
Method "B"	Method "C"		Method "B"	Method "C"		Method "A"	
1.48	1.61	1.57	1.48	1.51	1.50	1.52	1.54
1.43	1.43	1.40	1.52	1.47	1.47	1.53	...
1.51	1.54	1.50	1.53	1.53	1.53	1.54	1.53
						(Av. 1.53)	
1.48	1.55	1.56	1.53	1.55	1.52		
1.60	1.59	1.60	1.55	1.57	1.56		
1.53	1.51	1.51	1.49	1.56	1.53		
(Av. 1.51)	(Av. 1.53)		(Av. 1.52)	(Av. 1.53)			

[a] In each case six groups of three tablets were extracted by each method. Only one tetrazolium assay was done on each extract by method "B" but two tetrazolium assays were done on each extract by method "C."
[b] Ten tablets were ground to a powder and three aliquots of powder each representing three tablets were extracted separately. Two paper strips were run on each extract. One paper strip was damaged and could not be used.

Results obtained when tablet lot IA was stored at 70° without Humi-Caps in the bottles are plotted in Fig. 2. The values obtained by method "C" were corrected for the values of "prednisolone" found for the blank tablets. It can be seen that prednisolone in lot IA, stored at 70° in the absence of Humi-Caps, shows a linear degradation with time by all these methods. Linearity of such a plot indicates a constant degradation rate independent of concentration, i. e., pseudo zero order rate. The slopes of the lines shown in Fig. 2 were calculated by the method of "least squares" (9*b*) and the slopes were compared by calculating the value of t = difference in slopes/standard deviation of the difference (9*c*). The slopes of the lines do not differ at $P = 0.05$. However, there is obviously a bias in the results obtained by method "C."

The improvement in stability of prednisolone in tablets when the tablets are stored with a desiccant (Humi-Caps or Dri-Pax[3]) is noteworthy. In the 70° study with Humi-Caps the loss in potency which occurs during the first twenty-four hours may be ultimately caused by moisture in the tablet. The water is finally absorbed by the silica gel, and, once equilibrium is attained, no further loss in potency occurs. At 47° the improvement in stability when a desiccant is present is also evident.

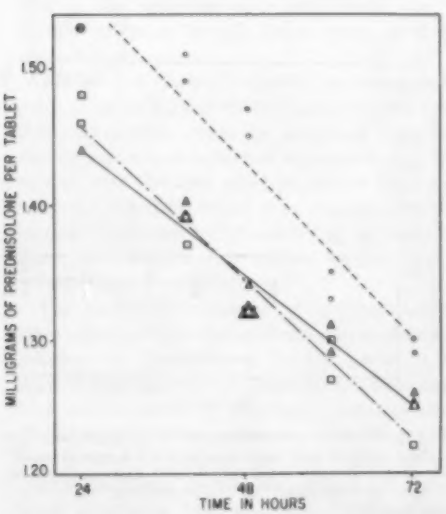

Fig. 2.—Plot of milligrams of prednisolone per tablet against time in hours for lot IA stored at 70° in sealed bottles with metal-liner caps without Humi-Caps. △—Method "A"; ⊡—method "B"; ⊙—method "C."

One and Two Month Stability Samples.—Table II is a tabulation of averages of duplicate assay values obtained by the three methods on aliquots of the same two month stability samples of Lots I and II and aliquots of the same one month stability samples of Lots IA and IIA. Tablets of the four lots were stored in sealed bottles under the conditions indicated in the table.

Evaluation of Data.—The average deviation of duplicate assays on 23 samples was 0.02 mg. of prednisolone for all methods; the 95% limits were 0.05 mg. of prednisolone per 1.5-mg. tablet which is an error of about 3%.

The data obtained by all three methods on a given group of samples were combined and an analysis of variance was made for each group. The results of these analyses are shown in Table III. When all three methods were included in the analyses a significant difference between methods and a significant interaction (samples × methods) was found for five out of the six groups of samples. The significant interactions were due mainly to the fact that method "C" gave consistently higher results than either methods "A" or "B" except on freshly prepared tablets. Hence, although method "C" had the same precision as methods "A" and "B" it did not have the same accuracy. Method "C" was concluded to be an unsatisfactory procedure since results obtained with the method appear to be markedly sample-dependent. There were indications that method "C" measured certain steroid degradation products which were not measured by methods "A" and "B."

Separate analyses of variance were performed for each group of samples using only the data obtained by methods "A" and "B." The results of these analyses are shown in Table III also. In only one

[3] Dri-Pax—A cloth bag enclosing approximately 3 Gm. of dried silica gel manufactured by Davison Chemical Company, Baltimore, Md.

TABLE II.—TABULATION OF AVERAGES OF DUPLICATE ASSAYS OBTAINED BY THREE DIFFERENT METHODS ON ALIQUOTS OF THE SAME STABILITY SAMPLES

Methods	Potency: Milligrams of Prednisolone Per Tablet — Samples: R. T.	R. T. + Humi-Caps	40°	40° + Humi-Caps	47°	47° + Dri-Pax or Humi-Caps
	Group III: Assays of Two Month Stability Samples of Lot I					
A	1.54	...	1.48	...	1.44	1.48 D-P
B	1.47	...	1.40	...	1.38	1.46
C	1.56	...	1.58	...	1.60	1.62
	Group IV: Assays of Two Month Stability Samples of Lot II					
A	1.58	...	1.33	...	1.21	1.35 D-P
B	1.47	...	1.28	...	1.13	1.33
C	1.56	...	1.38	...	1.29	1.46
	Group V: Assays of One Month Stability Samples of Lot IA					
A	...	1.55	...	1.42	...	1.50 H-C
B	...	1.51	...	1.45	...	1.53
C	...	1.53	...	1.55	...	1.66
	Group VI: Assays of One Month Stability Samples of Lot IIA					
A	...	1.49	...	1.47	...	1.36 H-C
B	...	1.49	...	1.43	...	1.42
C	...	1.56	...	1.56	...	1.56

TABLE III.—RESULTS OF ANALYSES OF VARIANCE OF ASSAY VALUES OBTAINED BY ALL THREE METHODS AND BY METHODS A AND B ONLY ON DIFFERENT GROUPS OF TABLETS

Data Analyzed	Analysis of All Three Methods — Probabilities of Significance of Difference — Among Samples	Among Methods[c]	Analysis of Methods "A" & "B" Only — Among Samples	Among Methods[c]
Group I[d]	N. S.[a]	0.001	0.05	N. S.
Group II[e]	0.001[b]	0.001	0.001	0.05
Group III[f]	N. S.[b]	0.01	0.001	0.001
Group IV[f]	0.001[b]	0.01	0.001	0.001
Group V[f]	N. S.[b]	N. S.	0.01	N. S.
Group VI[f]	N. S.[b]	0.05	N. S.[b]	0.05

[a] N. S. = Not significant.
[b] Interaction (samples × methods) significant.
[c] Part of the apparent difference between "methods" is due to "sampling" variation because the duplicate assays were duplicates only from the extract stage to spectrophotometric measurement and not from the powder aliquot to spectrophotometric measurement. No accurate estimate of the sampling error resulting from the use of different aliquots of ten crushed tablets of different tablet lots was made.
[d] See Fig. 1.
[e] See Fig. 2.
[f] Individual assay values from which averages in Table II calculated.

out of six groups of samples was there a significant interaction (samples × methods) and this was obviously due to the difference in results obtained by methods "A" and "B" on the tablet Lot IIA stored for one month at 47°.

There was a significant difference between methods "A" and "B" when applied to four out of the six groups of samples. However, from a practical standpoint the difference in results obtained by methods "A" and "B" are very small in all six groups.[3] The average deviation between results obtained by methods "A" and "B" is −0.02 mg. of prednisolone per tablet with a range of −0.11 to +0.06 mg. of prednisolone per 1.5-mg. tablet (deviations taken as average of duplicate assays by method "B" minus average of duplicate assays by method "A"). Expressing this in another way, one can say that potencies found by method "B" ranged from 93 to 104% with an average of 98.6% of the potencies found by method "A." It should be pointed out that such agreement between methods "A" and "B" may not apply to all tablet formulations or stability samples.

In Fig. 3, the ordinate Y, is the average potency found by duplicate assays with method "B" and the abscissa, X, is the average potency found by duplicate assays with method "A." There are twenty-four such averages by each method. The "least squares" line, $\hat{Y} = 0.908.X + 0.11$, calculated for these data was shown to have a slope not differing

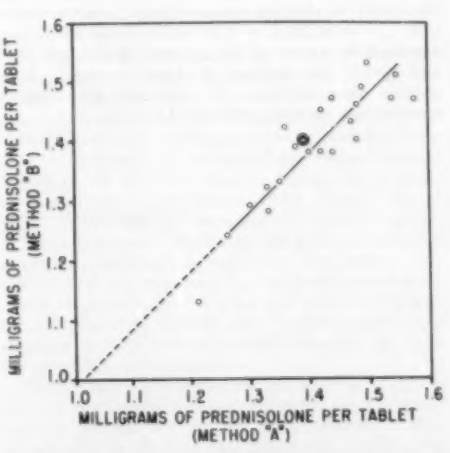

Fig. 3.—Correlation of assay results obtained by method "B" with results obtained by method "A." The solid line is calculated from the average slope, 0.986.

[3] See also footnote "c," Table III.

significantly from unity and an intercept not differing significantly from zero. The solid line in Fig. 3 represents the average slope, 0.986. Hence it may be concluded that results obtained by method "B" correlate well with results obtained by method "A."

In Fig. 4, the ordinate, Y' is the average potency found by duplicate assays with method "C" and the abscissa, X, is the average potency found by duplicate assays with method "A." There are

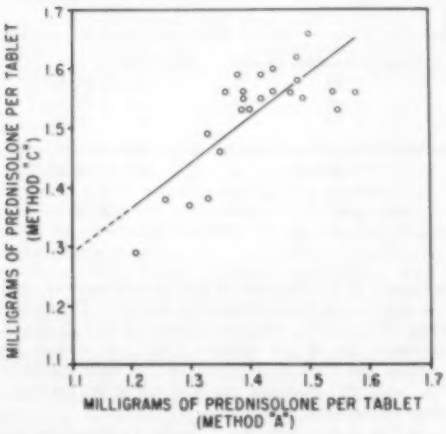

Fig. 4.—Correlation of assay results obtained by method "C" with results obtained by method "A." The line is calculated from the equation: $\hat{Y} = 0.739\,X + 0.48$ which is the fitted line obtained by means of the method outlined in reference (11) and by the method of "least squares."

twenty-four such averages by each method. The line shown on the plot was calculated from the equation: $\hat{Y}' = 0.739X + 0.48$ which is the fitted line obtained by means of the method of Bartlett (11) and also by the method of "least squares." It is obvious that method "C" does not give results correlating well with method "A."

SUMMARY AND CONCLUSIONS

1. A preliminary comparison of three methods of determining prednisolone in freshly made and aged tablets has been made.

2. A chloroform extraction-tetrazolium procedure and an alcohol extraction-tetrazolium procedure, are described in detail. The third method, a paper chromatographic procedure, has been described in detail in a previous communication.

3. The chloroform extraction-tetrazolium procedure gave comparable results to the paper chromatographic method for the samples checked. The former method, being less tedious, is the more desirable one for routine assays.

4. The alcohol extraction-tetrazolium procedure was found to be unsatisfactory and did not give results correlating well with the paper chromatographic method. The alcohol method gave high values for many of the stability samples although it compared well with the other methods when used for freshly prepared tablets.

5. The presence of a desiccant was shown to improve the stability of prednisolone in compressed tablets.

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A Study of the Titration of Organic Bases in Chlorobenzene with Acetous Perchloric Acid*

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Potentiometric titrations can be carried out in chlorobenzene by using glass and sleeve-type calomel electrodes and a Beckman Model GS pH Meter. Acetous perchloric acid is the most suitable titrant. Galvanometer response is rapid and no erratic galvanometer needle deflections are noted when this titrant is used. The optimum concentration of titrant at the end point appears to be approximately 20 parts titrant for every 100 parts of solution. Bases as weak as caffeine can be determined in this solvent but only with a limited degree of accuracy. A mixed solvent containing 20 per cent glacial acetic acid in chlorobenzene can be used for the titration of organic bases insoluble in chlorobenzene. Bromophenol blue is a suitable indicator for use in chlorobenzene. Neutral red, brilliant green, and methyl red can also be used in this solvent but indicator changes are not as satisfactory as with bromophenol blue.

TWENTY-FIVE YEARS ago, Conant, Hall, and WERNER (1–3) showed that many substances which exhibit no basic properties in water behave as relatively strong bases in glacial acetic acid. The analytical significance of their observations was not immediately realized and the technique of acid-base titrations in nonaqueous solvents was not generally accepted until after the second world war. The distinct advantage of this method, as compared with volumetric procedures carried out in aqueous media, made it particularly suited to the quality control of pharmaceuticals. The application of acid-base titrations to the analysis of pharmaceutically important compounds has been adequately reviewed by Pifer, Wollish, and Schmall (4). Reviews of a more general nature have been published by Riddick (5–7) and Ballczo (8, 9).

Research in this field has followed the pattern laid down by Conant, Hall, and Werner. The "super acids" appeared to offer the ultimate in base intensification and solubilization. A large number of papers, therefore, appeared in the literature dealing with the analysis of various organic bases in glacial acetic acid. It soon became evident that other solvents were superior to glacial acetic acid both in solubilizing ability and in base-strengthening power. Pifer, Wollish, and Schmall (10) showed that the addition of aprotic solvents to glacial acetic acid greatly increased the magnitude of the potential break at the end point in the potentiometric titration of very weak bases with acetous perchloric acid. Fritz (11) reported that many solvents gave as good or better results than did glacial acetic acid.

Chlorobenzene can be classified as an inert or aprotic solvent. Little ionization occurs in such solvents (8) and it is, therefore, an excellent medium for the reaction of basic compounds with acetous perchloric acid. The solvent, however, has not been extensively investigated. Fritz (11) reported the determination of several organic compounds in this solvent using either indicators or a glass and silver-silver chloride electrode combination to detect the end point of the titration. Rice, Zuffanti, and Luder (12) studied indicator color changes with "L-acids" in chlorobenzene. Deal, Weiss, and White (13) determined basic nitrogen compounds in asphalt by titration in a 50 per cent mixture of glacial acetic acid in chlorobenzene with acetous perchloric acid. They reported that potentiometric titration in chlorobenzene alone was not feasible.

EXPERIMENTAL

At the outset, it was necessary to establish proper conditions for potentiometric titration in chlorobenzene. Neither the glass and fiber-type calomel electrode combination nor the glass and silver-silver chloride electrode combination suggested by Fritz (11) were found to be suitable. Potentiometric titrations of several organic bases using these electrodes and a Beckman Model GS pH Meter were unsatisfactory because of erratic galvanometer response. Similarly, the gold and calomel electrode combination suggested by Zeidler (14) and Novak (15) was found to be unsatisfactory for use in chlorobenzene. A glass and glass sleeve-type calomel electrode combination showed satisfactory response during potentiometric titration of basic compounds in chlorobenzene.

Preliminary investigation of several titrants showed that acetous perchloric acid was the most suitable titrant for use in chlorobenzene. Perchloric acid in dioxane and perchloric acid in 20% glacial acetic acid in chlorobenzene were unsatisfactory because of erratic galvanometer response. Diphenyl phosphate, a titrant used by Davis and Hetzer (16) for titrations in aprotic solvents, was

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unsatisfactory because of its poor solubility in chlorobenzene.

These exploratory potentiometric titrations of several organic bases in chlorobenzene established that satisfactory titration curves could be obtained using glass and sleeve-type calomel electrodes, a Beckman Model GS pH Meter, and acetous perchloric acid as titrant. These observations confirm literature reports that the glass and sleeve-type calomel electrode combination and acetous perchloric acid are generally the most satisfactory electrode combination and titrant for use in alkalimetric titrations in nonaqueous media.

Titration Assembly.—A Beckman Blue Label Glass Electrode No. 1190-80 and a Beckman Sleeve-Type Calomel Electrode No. 1170-71 were inserted into a Beckman Electrode Holder and placed in the solution being titrated. The Beckman Electrode Holder provided standard electrode spacing throughout the titrations. Decreasing this distance between electrodes did not appear to have any beneficial effect on the nature of the potentiometric titration. The electrodes were connected to a Beckman Model GS pH Meter, Serial No. 97182, and the potentiometric titration was carried out with the sensitivity switch in position *A* and the pH-Mv switch in position +Mv. During the titration, the acetous perchloric acid and the solution were mixed with a glass stirring rod. A magnetic stirrer may be used but extreme galvanometer deflection results which necessitates disconnection of the stirrer when millivolt readings are being taken.

Solvent Purification.—Approximately 10 Gm. of anhydrous granular calcium chloride were added to 500 ml. of chlorobenzene (Eastman reagent grade) and allowed to stand for twenty-four hours. The solvent was distilled from an all glass apparatus and the distillate collected when the boiling point became constant. The procedure was repeated and after the second distillation the solvent was considered ready for use. The observed boiling point was 129.5°. Literature values of 130.25–132° have been reported (17). Refractive index values agreed satisfactorily with the values reported in the literature.

Choice and Purity of Organic Bases Titrated.—Nine organic compounds, with pKb values in water ranging from 4.35 to 14.00, were studied during the course of this investigation. In addition, salts of two organic bases and one salt of an organic acid were included in the list of compounds titrated during the investigation. The organic bases and salts chosen were: atropine, strychnine, ephedrine, alanine, cinchonine, amidopyrine, benzocaine, caffeine, theobromine, atropine sulfate, ephedrine sulfate, and potassium acid phthalate. All the organic bases were dried in an oven set at 100° or over phosphorous pentoxide depending on the melting point of the compound.

The melting points of the organic bases were determined using either a Purdue melting point bath or a Fisher-John melting point apparatus. If the melting point of the compound did not agree with the value reported in the literature, the organic base was recrystallized by dissolving in a minimal amount of alcohol and precipitating with an excess of distilled water. Atropine sulfate was recrystallized from dioxane.

Titrant.—The acetous perchloric acid used in this investigation was prepared and standardized according to the procedure given by Seaman and Allen (18). The acid was added to the solution of the organic base in chlorobenzene or 20% glacial acetic acid in chlorobenzene from a 5.0-ml. buret calibrated to 0.02 ml. Increments of 0.02 ml. were added to the solution in the end point region both in potentiometric and indicator titrations. No attempt was made to add less than 0.02 ml. of titrant even though end point detection to 0.01 ml. is possible in many acid-base titrations in nonaqueous media.

Titrations in Chlorobenzene.—One milliequivalent weight of the organic base was transferred quantitatively with the aid of chlorobenzene to a previously calibrated 100-ml. volumetric flask. The solution was diluted to 100.0 ml. with chlorobenzene, mixed, and 25.0-ml. aliquots were measured from a buret for potentiometric titration with 0.0460 *N* acetous perchloric acid. The same procedure was used for indicator titrations in chlorobenzene.

This procedure provided a means of titrating 0.25 milliequivalent weight of the organic base without resorting to the weighing of semimicro quantities of material. Moreover, a direct comparison of all titration curves could be made since the ratio of solution to titrant was approximately the same in all titrations. Each titration required approximately 5.4 ml. of acetous perchloric acid and the ratio of titrant to solution at the end point was, therefore, 21.6 to 100.

Titrations in 20% Glacial Acetic Acid in Chlorobenzene.—The samples were prepared for titration in a manner similar to that given above. One milliequivalent weight of the organic base was transferred to a 100-ml. volumetric flask with the aid of 20.0 ml. of glacial acetic acid and sufficient chlorobenzene was added to make the final volume 100.0 ml. Titrations were carried out on 50-ml. aliquots of the solution. This, however, did not change the ratio of titrant to solution since both the volumes of titrant and solution were doubled.

Titrations were carried out in this mixed solvent system for two reasons:

(*a*) It has been reported in the literature that the addition of solvents of low dielectric constant to solutions of weak bases in minimal amounts of glacial acetic acid enhanced the potential change at the end point (10). This phenomenon occurs even when the glacial acetic acid content of the solution is further increased by the addition of acetous perchloric acid. Potentiometric titrations in 20% glacial acetic acid in chlorobenzene would confirm this observation.

(*b*) Many organic bases are readily soluble in glacial acetic acid. Since it was expected that several of the organic bases used in this investigation would be insoluble in chlorobenzene, some other method of solubilization had to be used that would retain a solvent system consisting mainly of chlorobenzene. Glacial acetic acid was found to be the most satisfactory solubilizing agent investigated. It provided a stable titration medium for potentiometric titration and, at the same time, solubilized the majority of the organic bases used in this investigation.

Other solubilizing agents were investigated in this phase of the work. It has been reported in the literature that morphine and codeine phosphate can be easily solubilized in chloroform by the addition of 1% and 5% phenol, respectively (19, 20). Addition of 5% phenol to the chlorobenzene was found to be unsuitable because of erratic galvanometer response during potentiometric titration. In addition, the solubility of the organic base was not materially increased.

DISCUSSION

The solubility of the organic bases used in this investigation in chlorobenzene or 20% glacial acetic acid in chlorobenzene was better than expected. Only alanine was found to be insoluble in both solvent systems. Theobromine and cinchonine were insoluble in chlorobenzene but, at the same time, their solubility in glacial acetic acid was poor. Heat was used to solubilize these bases in glacial acetic acid prior to the addition of chlorobenzene. The alkaloidal salts and the salt of the organic acid used in this investigation were solubilized in 20% glacial acetic acid in chlorobenzene without any difficulty.

On the basis of experimental observations, it would appear that the titrant should be approximately 0.05 *N* in order to carry out a satisfactory potentiometric titration. In addition, the ratio of titrant to solution at the end point should be approximately 20 to 100. The addition of larger quantities of titrant increased the stability of the solvent system during the potentiometric titration but, at the same time, decreases the value of the factor dE/dV at the end point. The ratio of 20 parts of titrant to 100 parts of solution is not critical. Several titrations were performed in which the titration at the end point was approximately 5 to 100. Although it is possible to carry out titrations at this level, the solvent system is not as stable as with higher titrant to solution ratios.

Typical titration curves for the weak base amidopyrine in chlorobenzene and in 20% glacial acetic acid in chlorobenzene are shown in Fig. 1.

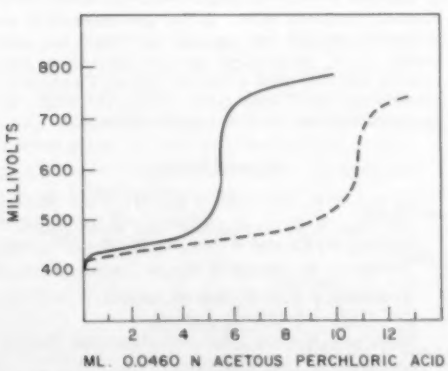

Fig. 1.—Titration curve for amidopyrine in chlorobenzene ——— and in 20% glacial acetic acid in chlorobenzene – – –.

The titration curves for the stronger bases showed greater potential changes at the end point while the potential changes for the weaker bases were less pronounced. As an example, in chlorobenzene the value of the factor dE/dV at the end point for strychnine, a moderately strong base in nonaqueous media, was found to be approximately 4,000 for 0.02 ml. of titrant. Amidopyrine, a weaker base in nonaqueous media, showed a potential change of approximately 3,000 for 0.02 ml. of titrant. The value of the factor dE/dV at the end point for benzocaine, a very weak base, was found to be approximately 400 for 0.04 ml. of titrant.

In this respect, there does not appear to be any pattern for potential changes at the end point in chlorobenzene. Bases with pKb values in water of less than 10 show essentially the same magnitude of change at the end point. The very weak bases, on the other hand, differ greatly from those with pKb values in water of less than 10. For these very weak bases, the values of the factor dE/dV at the end point range from 2 to 10% of the values observed for the stronger bases. Those compounds with pKb values in water of more than 10 can be determined only by using exacting techniques and careful millivolt measurements. Other solvents would be preferable for the determination of these compounds.

Each organic base was titrated in triplicate with acetous perchloric acid and the titration curves compared. The titration curves for the stronger bases were essentially identical. Variations were observed in the titration curves of the weaker bases. Either a shift of the titration curve upward or a decrease in millivolt readings after the end point region may be expected. These variations in the titration curves of any one compound do not interfere with the detection of the end point.

Perchlorate precipitates form during the titration of organic bases in chlorobenzene. During the titration of atropine, the solution turns murky but no true precipitate forms. Ephedrine does not form an insoluble perchlorate salt. These precipitates do not interfere with the titration of the organic compound. There is some evidence that the formation of crystalline precipitates during the titration aids in the detection of the indicator end points of very weak bases (21).

Bromophenol blue, methyl red, neutral red, brilliant green, and diazoamidobenzene were found to be suitable for the detection of the end point during the titration of bases stronger than amidopyrine. The color changes of these indicators at the end point are shown in Table I. Indicator color changes for bases having pKb values in water

TABLE I.—INDICATOR COLOR CHANGES FOR STRONG BASES TITRATED IN CHLOROBENZENE WITH ACETOUS PERCHLORIC ACID

Indicator	Base Color	Acid Color
Bromophenol blue	Yellow[a]	Colorless
Neutral red	Orange[a]	Purple
Brilliant green	Green	Yellow-green
Methyl red	Orange	Red
Diazoamidobenzene	Yellow-green	Orange

[a] The initial color of this indicator varies with the strength of the base.

of more than 10 were gradual and the end point could not be detected. Diazoamidobenzene is suitable only for the titration of atropine and ephedrine. The formation of perchlorate precipitates obscures the color change at the end point and overestimation of the organic base results. Of the five indicators investigated, bromophenol blue appears to be the most satisfactory indicator for alkalimetric titrations in chlorobenzene.

Bromophenol blue and neutral red exhibited unique characteristics in this solvent. The initial colors of these indicators in the solution of the organic base in chlorobenzene appeared to signal the strength of the compound. Addition of bromophenol blue to solutions of ephedrine or atropine in chlorobenzene resulted in the formation of a purple color. Solutions of strychnine in chlorobenzene exhibited rose-red colors when the indicator was added. Yellow colors resulted when the indicator was added to solutions of amidopyrine, benzocaine, or caffeine in chlorobenzene. The intensity of the yellow color, however, varied with the strength of the base. A bright yellow color was observed when amidopyrine was the organic base under investigation and pale yellow colors were observed with chlorobenzene solutions of caffeine and benzocaine. A similar phenomenon was observed with neutral red indicator. This indicator, however, did not distinguish between atropine, ephedrine, and strychnine. Solutions of these three bases all showed the same yellow color. Pink indicator colors were observed in chlorobenzene solutions of caffeine and benzocaine. Amidopyrine showed an initial color of intermediate hue.

The accuracy and precision of either indicator or potentiometric titrations in chlorobenzene is as good as that observed in the titration of strong bases in aqueous media. The per cent recoveries of the weighed amounts of the organic bases were satisfactory. Six potentiometric titrations were carried out on samples of atropine in chlorobenzene. At the 0.1 milliequivalent weight level, the per cent recovery of the alkaloid was 100.51% and the standard deviation was equal to 1.12%. At the 0.2 milliequivalent weight level, 99.71% of the alkaloid was recovered and the standard deviation was calculated to be 0.55%. This would indicate that the procedure gives more precise results when slightly larger quantities of the organic base are titrated.

All the organic bases, with the exception of alanine, were also titrated in 20% glacial acetic acid in chlorobenzene. From the potentiometric titrations performed in this solvent system, three conclusions may be reached:

(*a*) The addition of large amounts of glacial acetic acid to chlorobenzene are to be avoided. In all cases, the magnitude of the potential break at the end point was sharply decreased from the value observed in chlorobenzene alone. The magnitude of the potential break at the end point for all compounds titrated in this investigation both in chlorobenzene and in 20% glacial acetic acid in chlorobenzene are shown in Fig. 2. No absolute values are reported but only relative values based on 0.1 ml. of titrant. As an example, if the value of the factor dE/dV at the end point was 4,000 for 0.02 ml. of titrant, the value reported in this figure would be $4{,}000 \times 0.10/0.02$ or 20,000.

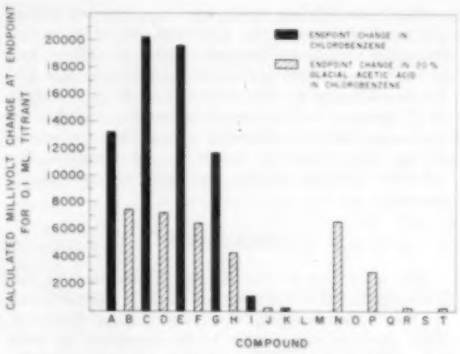

Fig. 2.—Relative changes at end point for atropine (AB), strychnine (CD), ephedrine (EF), amidopyrine (GH), benzocaine (IJ), caffeine (KL), potassium acid phthalate (MN), cinchonine (OP), ephedrine sulfate (QR), and atropine sulfate (ST). MOQS: insoluble in chlorobenzene.

(*b*) The titrations carried out in 20% glacial acetic acid in chlorobenzene confirm literature reports that the magnitude of the potential break at the end point is increased by the addition of large amounts of aprotic solvents to minimal amounts of glacial acetic acid (10).

(*c*) Alkaloidal salts are insoluble in chlorobenzene. They are, however, soluble in 20% glacial acetic acid in chlorobenzene and can be determined accurately by titration with acetous perchloric acid. The determination of atropine sulfate and ephedrine sulfate have been previously reported in the literature (22, 23). From personal observations and from literature reports (24), it would appear that the reaction of sulfates with perchloric acid in a glacial acetic acid medium are not stoichiometric. By the addition of chlorobenzene to a glacial acetic acid, stoichiometric results can be obtained. Potential breaks for the two alkaloidal salts are not too great, but the end point can be determined easily.

From this discussion of the titration of organic bases in chlorobenzene, it is evident that the solvent is suitable for use in alkalimetric titrations with acetous perchloric acid. Either potentiometric or indicator methods can be used to detect the end point. Very weak bases can be titrated in this solvent but only with a limited degree of accuracy. Determination of such bases, therefore, would be better carried out in other organic solvents.

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Chronic Oral Toxicity of Aerated Silica Gel*

By CHANDRAKANT H. DESAI, ALLAN BURKMAN, and RUPERT SALISBURY

It would appear that aerated silica gel of the type used is not entirely inert in rats since there appears to be a significant change in the body associated with its continued administration. A comprehensive investigation of chronic oral toxicity of aerated silica gel seems necessary before its oral use can be approved.

IN THE COURSE of a series of investigations testing the use of a relatively inert, insoluble material in the prevention of caking in pharmaceutical suspensions, aerated silica gel[1] showed some promise and chronic oral toxicity tests were undertaken to determine the feasibility of oral administration of large amounts of aerated silica gel.

Dried silica gel is a tasteless, odorless, relatively inert powder which has an enormous surface area (the material used was of a particularly fine particle size, from 3 to 5 microns in diameter) and is claimed to be capable of adsorbing toxins; it was used for this purpose locally in severe burn cases and internally in the treatment of certain types of gastroenteritis (1).

Intranvenously administered injections of colloidal silica caused rapid death in mice according to Dale and King (2). Injection of 5 mg. of finely powdered silica gel was injected into each renal artery of rats with a resulting hypertension of many months' duration because of diffuse intrarenal fibrous tissue action (3).

In studying the toxicity of silica in various textbooks of toxicology (4, 5) it becomes apparent that the greatest interest has been in industrial silicosis due to the mechanical irritation produced by inhalation rather than by ingestion of the material. Since aerated silica gel has properties which have possibilities for internal application and since no work has been done on its oral chronic toxicity, this study was undertaken to evaluate the chronic oral toxicity of aerated silica gel.

EXPERIMENTAL

Male and female, Sprague-Dawley, young adult rats were utilized in the study. Throughout the experiment they were fed Purina Laboratory Chow and water *ad libitum*. Test group I consisting of 12 rats received a daily oral dose of aerated silica gel equivalent to 5.71 mg./Kg. for a period of eight weeks. Test group II (12 rats) received a daily oral dose of aerated silica gel equivalent to 17.14 mg./Kg. A control group of 12 rats received a dose of methylcellulose equivalent to 5.71 mg./Kg.

The 36 rats used were approximately of the same age and weight. Oral administration was by stomach tube and syringe. All the rats were weighed initially and then weekly. Doses were calculated on a weekly basis and administered daily.

RESULTS AND DISCUSSION

The eight weeks of daily oral administration of aerated silica gel in different concentrations and the use of methylcellulose as a control did not produce a single untoward reaction in the experimental animals. All remained normal with no signs of abnormality. During the eight week experiment it was observed that the rate of gain in weight in the experimental groups was not as great as that in the control groups (Fig. 1). Due to the rather small groups of animals used and in view of the more significant pathological findings no significance is attached to the change in rate of gain in weight at this time.

Post-mortem examination revealed abnormal larger nodules on the intestine of the test rats and not on the control group. There were more nodules in the test rats which received the larger amounts of aerated silica gel. All other factors were quite normal.

* Received February 2, 1958, from the College of Pharmacy, The Ohio State University, Columbus.
Abstract of a portion of a dissertation presented to the Graduate School of The Ohio State University by Chandrakant H. Desai in partial fulfillment of the requirements for the degree of Master of Science.
[1] Kindly supplied by the Monsanto Chemical Co. under the trade name, Santocel C.

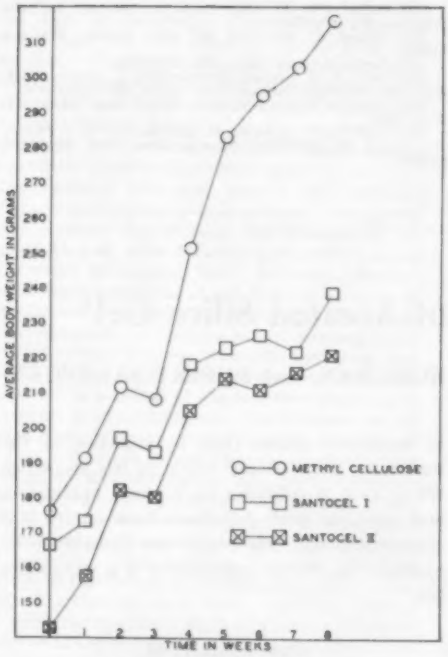

Fig. 1.—Growth curves.

Figure 2.

Gross Description of Intestinal Nodules.—Palpable masses of firm tissue were on the external surface opposing the area of mesentery attachment of the gut wall (Fig. 2). Numerous dense opaque disk-shaped masses devoid of villi characterized the nodule when observed within the intestinal lumen (Fig. 3). They were externally similar to the tubercle-like lesions of tuberculosis or neoplastic disease; lesions larger than those of tuberculosis but less diffuse were limited only to the gut wall.

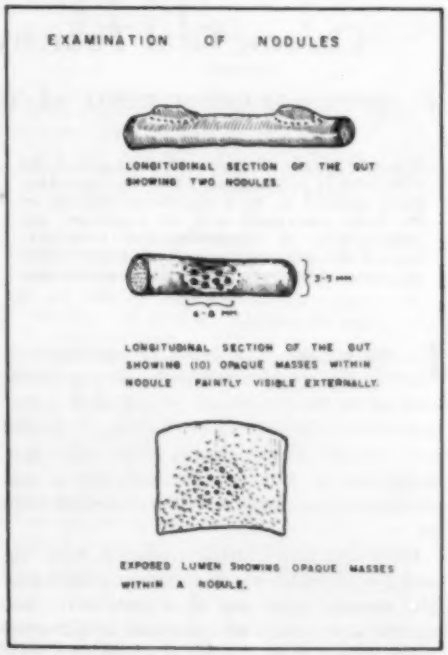
EXAMINATION OF NODULES
LONGITUDINAL SECTION OF THE GUT SHOWING TWO NODULES.
3-5 mm
LONGITUDINAL SECTION OF THE GUT SHOWING (10) OPAQUE MASSES WITHIN NODULE FAINTLY VISIBLE EXTERNALLY.
EXPOSED LUMEN SHOWING OPAQUE MASSES WITHIN A NODULE.

Figure 3.

Microscopic Description.—The major portion of the nodule consists of masses of lymphoid connective tissue.

Etiology of Nodules.—The position of the nodules and their etiologic characteristics suggest that they are very much enlarged Payer's patches. The enlargement in turn is a typical response to foreign body invasion, possibly caused by very small insoluble silica gel particles imbedded in tissue with the formation of nodules through foreign body reaction.

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The Effect of Some Antihistamines upon the Respiration of Rat Heart Homogenates*

By DORIS E. McKERSIE and HAROLD C. HEIM

Conventional manometric techniques were used to investigate the effects of some antihistamines upon the respiration of rat heart homogenates. The study was undertaken because it has been reported that these drugs exert effects on cardiac arrhythmias but little is known relative to the mechanisms by which these effects are produced. The antihistamines which were studied were promethazine HCl, phenindamine tartrate, methapyrilene HCl, antazoline HCl, diphenhydramine HCl, tripellenamine HCl, prophenpyridamine maleate, and pyrilamine maleate. Each of these drugs, in 0.007 *M* concentration, inhibited endogenous respiration as well as respiration in the presence of glucose or pyruvate. Both promethazine HCl and phenindamine tartrate markedly inhibited respiration in the presence of succinate. None of the other antihistamines included in the study produced any significant effect upon respiration in the presence of succinate. The effects elicited by the antihistamines could not be significantly altered by the addition of histamine to the Warburg vessels.

SEVERAL INVESTIGATORS have reported that certain antihistamine drugs exert cardiodepressant activity. It has been reported (1) that diphenhydramine exerts an effect similar to that of quinidine in converting an auricular fibrillation to a normal sinus rhythm in dogs previously sensitized to epinephrine by the administration of chloroform. It has been shown (2) that some antihistamines, at rather high dosage levels, were effective in preventing ventricular arrhythmias induced in rats by the administration of calcium chloride and that in even higher dosages they were effective in overcoming the arrhythmias induced by the previous administration of aconitine. Reports of other studies have appeared in which it has been shown that diphenhydramine is more effective than quinidine in abolishing the fibrillation produced in the dog heart by the administration of acetylcholine (3).

It appears that little attention has been devoted to the effects of the antihistamines on respiratory processes of the heart. In view of the fact that several reports indicate that the antihistamines exhibit some cardiodepressant activity it seemed of interest to study the effects elicited upon the respiration of heart homogenates by certain drugs in this group.

EXPERIMENTAL

The hearts from healthy, adult Sprague-Dawley rats of either sex and weighing between 200 and 250 Gm. were used in all experiments. The animals were killed by a blow to the head after which the hearts were immediately removed and placed in a beaker of ice-cold isotonic sucrose (0.25 *M*) solution. Each heart was allowed to beat for a few seconds in the isotonic sucrose solution in order to remove as much blood as possible. The hearts were then cut open and blotted dry on filter paper after which they were weighed and placed in a glass homogenizer containing cold isotonic sucrose solution. The homogenates were prepared so that each ml. of homogenate contained 160 mg. fresh heart tissue. No longer than twenty minutes elapsed from the time the animals were killed to the start of the experiment. Oxygen consumption was determined by the direct method of Warburg. When endogenous respiration was being studied 1.9 ml. of buffer, 0.4 ml. of the antihistamine solution or 0.4 ml. of distilled water, and 0.5 ml. of the freshly prepared homogenate were added to the main compartment of Warburg vessels of approximately 15-ml. capacity. The center well contained 0.2 ml. of 10% potassium hydroxide and a pleated strip of filter paper. When respiration in the presence of substrates was being studied 1.6 ml. of buffer and 0.3 ml. of the particular substrate were added to the main compartment in place of 1.9 ml. of buffer. In all experiments the total volume of the flask contents was 3.0 ml. The buffer used in all experiments was 0.1 *M* phosphate, pH 7.3.

The antihistamines under investigation were pyrilamine maleate (Neoantergan), prophenpyridamine maleate (Trimeton), tripellenamine HCl (Pyribenzamine), diphenhydramine HCl (Benadryl), promethazine HCl (Phenergan), phenindamine tartrate (Thephorin), methapyrilene HCl (Thenylene), and antazoline HCl (Antistine). The substrates studied were pyruvate, succinate, and glucose. The final concentration of each substrate was 0.01 *M* and the final concentration of each antihistamine was 0.007 *M*. Eight hearts were used for each experiment and duplicate flasks were run in every instance.

After a ten minute equilibration period the manometers were closed and readings were taken after an hour had elapsed. The temperature of the bath was 37° and the gas phase was air. All pH determinations were made with a glass electrode instrument.

Standard errors were calculated from the equation $\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$ and if the condition $M_1 - M_2 > 2\sqrt{\epsilon_1^2 + \epsilon_2^2}$ was found to be true, the results were considered to be significant.

* Received December 2, 1957, from the University of Colorado, College of Pharmacy, Boulder.

TABLE I.

Substrate	Antihistamine	mm.³ O_2 Uptake in 60 Min./100 mg. Fresh Tissue	Inhibition (−) or Stimulation (+), %
None	None	23 ± 0.85[a]	
None	Diphenhydramine HCl	13 ± 0.80	−44
Glucose	None	15 ± 0.75	
Glucose	Diphenhydramine HCl	10 ± 0.87	−33
Pyruvate	None	31 ± 0.94	
Pyruvate	Diphenhydramine HCl	14 ± 0.55	−55
Succinate	None	401 ± 5.89	
Succinate	Diphenhydramine HCl	387 ± 4.72	+4
None	None	21 ± 0.91	
None	Tripellenamine HCl	13 ± 0.74	−42
Glucose	None	18 ± 0.84	
Glucose	Tripellenamine HCl	11 ± 0.64	−39
Pyruvate	None	33 ± 1.18	
Pyruvate	Tripellenamine HCl	11 ± 0.72	−67
Succinate	None	432 ± 3.88	
Succinate	Tripellenamine HCl	438 ± 4.04	+1
None	None	18 ± 1.13	
None	Prophenpyridamine Maleate	10 ± 0.80	−45
Glucose	None	18 ± 1.13	
Glucose	Prophenpyridamine Maleate	13 ± 1.09	−38
Pyruvate	None	33 ± 1.24	
Pyruvate	Prophenpyridamine Maleate	15 ± 1.22	−55
Succinate	None	441 ± 5.49	
Succinate	Prophenpyridamine Maleate	436 ± 5.28	−1
None	None	21 ± 0.89	
None	Pyrilamine Maleate	11 ± 0.66	−48
Glucose	None	23 ± 0.99	
Glucose	Pyrilamine Maleate	11 ± 0.69	−52
Pyruvate	None	33 ± 1.15	
Pyruvate	Pyrilamine Maleate	11 ± 0.66	−67
Succinate	None	414 ± 4.85	
Succinate	Pyrilamine Maleate	444 ± 8.06	+7
None	None	21 ± 1.07	
None	Promethazine HCl	11 ± 1.19	−48
Glucose	None	21 ± 0.74	
Glucose	Promethazine HCl	13 ± 0.98	−42
Pyruvate	None	34 ± 1.25	
Pyruvate	Promethazine HCl	13 ± 1.09	−62
Succinate	None	431 ± 3.73	
Succinate	Promethazine HCl	11 ± 1.09	−97
None	None	21 ± 1.14	
None	Phenindamine Tartrate	11 ± 0.95	−48
Glucose	None	25 ± 1.24	
Glucose	Phenindamine Tartrate	15 ± 1.03	−40
Pyruvate	None	35 ± 0.92	
Pyruvate	Phenindamine Tartrate	20 ± 1.42	−43
Succinate	None	414 ± 3.74	
Succinate	Phenindamine Tartrate	24 ± 2.08	−94
None	None	24 ± 1.32	
None	Methapyrilene HCl	13 ± 0.77	−46
Glucose	None	19 ± 0.89	
Glucose	Methapyrilene HCl	13 ± 0.61	−32
Pyruvate	None	36 ± 1.68	
Pyruvate	Methapyrilene HCl	14 ± 1.19	−61
Succinate	None	414 ± 5.40	
Succinate	Methapyrilene HCl	433 ± 6.25	+5
None	None	23 ± 1.48	
None	Antazoline HCl	14 ± 0.62	−39
Glucose	None	25 ± 1.27	
Glucose	Antazoline HCl	15 ± 1.37	−40
Pyruvate	None	38 ± 2.10	
Pyruvate	Antazoline HCl	16 ± 1.15	−58
Succinate	None	409 ± 5.94	
Succinate	Antazoline HCl	431 ± 7.11	+5

[a] Standard error.

RESULTS AND DISCUSSION

The effects elicited by the antihistamines upon the endogenous respiration and upon respiration in the presence of glucose, pyruvate, and succinate are shown in the Table. The endogenous respiration and respiration in the presence of glucose or pyruvate was significantly inhibited by all the antihistamines included in this study. In general, the greatest inhibition was produced with pyruvate as the substrate. With succinate as the substrate six of the antihistamines produced only an insignificant change in respiration which, in some instances, was a slight inhibition and in others a slight stimulation. With two of the antihistamines, however, (phenindamine tartrate and promethazine HCl) respiration in the presence of succinate was very markedly inhibited. Both phenindamine and promethazine differ structurally to a considerable degree from the other antihistamines in that promethazine contains a phenothiazine nucleus while phenindamine may be thought of as a phenylindene derivative with a dimethylamino-ethyl side chain which has been cyclized to form a tetrahydropyridine ring.

Because the antihistamines are thought to elicit their principal therapeutic effects by blocking histamine from cellular receptor sites, it was decided to study the effect produced on respiration of the homogenates in the presence of both histamine and the antihistamines. It was found that the effects produced by the antihistamines were not reversed by histamine. Histamine, at a final concentration of 0.005 *M*, produced a slight augmentation of oxygen uptake in the presence of all three substrates and also seemed to produce a somewhat more significant stimulation of the endogenous respiration. When histamine and the antihistamines were both added to the homogenates, however, the respiration was inhibited to essentially the same degree as when the histamine was omitted. Respiration in the presence of succinate was not inhibited by a combination of histamine and antihistamine except in those flasks to which phenindamine or promethazine were added. In this latter instance the inhibition of succinate oxidation was the same whether or not histamine was added to the flasks. These results would indicate that the effects of the antihistamines on the respiration of rat heart is mediated through mechanisms other than those involving histamine.

In a previous study (4) it was shown that emetine, a cardiotoxic agent, inhibited respiration of heart homogenates both in the presence and in the absence of glucose or pyruvate, and that emetine stimulated respiration in the presence of succinate. The effect of the antihistamines upon respiration of heart, *in vitro*, seems to be similar to some extent to the effects produced by emetine, yet the administration of the antihistamines does not evoke the cardiotoxic effects which often follow the prolonged administration of emetine (5). Emetine, however, is a cumulative drug while the antihistamines are rather rapidly metabolized or excreted (6).

It would seem conceivable that the relatively mild cardiodepressant effects elicited by some of the antihistamines might be mediated through an inhibition of the oxidation of metabolites other than succinate, thereby increasing the negative inotropic effect of succinate upon the myocardium. It has been reported (7) that succinate elicits a strong negative inotropic effect upon guinea pig heart slices, and that after poisoning with chlorobutanol or pentobarbital the heart slices were even more susceptible to this effect. Other investigators (8) have reported that, under conditions of reduced oxygen tension, succinate markedly increases the rate of oxygen consumption of dog myocardium and that succinate competes with other metabolites for oxygen, resulting in a lower rate of oxidation of the other normally occurring metabolites. The energy produced by the oxidation of succinate is thought to be incapable of increasing the rates of other metabolic processes which were depressed under conditions of low oxygen tension.

SUMMARY

1. The effects produced by eight different antihistamines upon the respiration of rat heart homogenates has been studied.

2. All the antihistamines studied produced inhibition of endogenous respiration and of respiration in the presence of glucose or pyruvate. In general, inhibition was greatest with pyruvate as the substrate.

3. Six of the antihistamines produced little, if any, effect on respiration in the presence of succinate. Two of the anthistamines (promethazine HCl and phenindamine tartrate) produced a very marked inhibition of respiration in the presence of succinate.

4. The effects on respiration were not significantly altered by the addition of histamine.

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Enzymatic Decomposition of Digitalis Glycosides II*

By OLE GISVOLD

THE RESULTS of previous investigations (1) in our laboratories have shown that when the fresh leaves of *Digitalis lanata*, *Digitalis lutea*, and *Digitalis ferruginea* were disintegrated quickly in the presence of water or dilute methanol or ethanol, heated for a brief period and then filtered, we were able to isolate or detect the desglucoglycosides such as acetyldigoxin, acetyldigitoxin, digitoxin, digoxin, and gitoxin. Apparently this technique leads to the ready enzymatic hydrolysis of the terminal glucose residue found in the native glycosides. These studies have been extended in a qualitative way to the following digitalis species, i. e., *Digitalis purpurea*, *Digitalis mertonensis*, *Digitalis amandiana*, and *Digitalis siberica*. In all the digitalis species investigated the desglucoglycosides were obtained. In some cases this technique led to the ready isolation of the glycosides in a crystalline state. It should be recorded that in a model experiment, adjustment of the water or aqueous methanol or ethanol to a pH of 7 with a phosphate buffer did not alter the rapid enzymatic removal of the terminal glucose residue of certain digitalis glycosides.

Stoll's (2) enzyme inhibiting technique when applied to the detection of the native digitalis glycosides is time consuming and the final preparation is contaminated with many pigments. A greatly simplified technique has been developed which in my hands led to the ready detection of the native glycosides. It differed only from the brief enzymatic studies in that the fresh leaves were first boiled for five minutes in either water, dilute aqueous methanol or ethanol, phosphate buffered (pH 7) water or phosphate buffered (pH 7) dilute aqueous methanol or ethanol. Boiling for short periods of time apparently inactivates the carbohydrase or carbohydrases that enzymatically cleave the terminal glucose residue present in the native glycosides. In all the digitalis species investigated, Stoll's native glycosides could be detected. Buffered solutions were used in order to minimize the possibility of the hydrolysis of the glycosides. This, because the aqueous extracts of the fresh leaves of the digitalis species are below a pH of seven and the digitalis glycosides that have a 2-desoxy sugar directly attached to the C_3-OH group of the aglycon are sensitive to acid hydrolysis even in the cold.

* Received November 15, 1957. From the College of Pharmacy, University of Minnesota, Minneapolis 14.

The results of my investigations serve to emphasize further that the recording of the nature of the glycosides found in a given digitalis species should always be accompanied by an exact history of the plant material that was used. It becomes very clear that for isolation purposes of the cardiac glycosides found in the digitalis species the method of handling, i. e., collection, storage, etc., and techniques of extraction determine to a great degree the products that will be obtained. These factors also are extremely important when the whole leaf or extracts thereof are used for oral purposes. This because the amount of absorption that is obtained by the oral route is to a great degree dependent upon the composition of the glycosides that are present, i.e., lanatoside C *versus* digoxin or acetyldigoxin or purpurea glycoside-A *versus* digitoxin or more strikingly gitaloxin *versus* gitoxin, etc.

A new solvent system (System II) has been developed that readily separates some of the native glycosides. It can be used by the ascending or descending techniques. Separations can be made in two to twenty-four hours depending on the composition of the glycosides and the degree of separation desired. This system also can be used to good advantage with the desglucosides. Systems I and II give good separation of the genins which previously (3) has been accomplished by another system on formamide impregnated paper. Systems requiring equilibration that will separate the genins also have been reported (4).

EXPERIMENTAL

Paper Chromatography.—Whatman No. 1 paper in sizes approximately 14 cm. x 26 cm. were used in the ascending technique and those used in the descending technique were 14 cm. x 38 cm.

Solvent System I.—This system is the same as that previously described (5). It consists of methyl isobutyl ketone-isopropyl ether, 100:25 (by volume), saturated with formamide. The stationary phase, formamide, was applied to the paper via a 30% solution in acetone. No equilibration is necessary.

Solvent System II.—This system was prepared from methyl isobutyl ketone, isopropyl ether, tetrahydrofuran, and formamide in the ratio of 40:10:15:15. The stationary phase is the same as that used in solvent system II. No equilibration is necessary.

The reagents that were used in the paper chromatographic studies were purified as previously described

(1). Isopropyl ether reagent grade was passed through a column of aluminum oxide to remove the peroxides. Tetrahydrofuran also was passed through the same kind of column and then distilled and the fraction boiling at 65° was collected and used. The positions of the glycosides on the paper were detected by the use of the Raymond reagent (6) or by Jensen's trichloroacetic acid reagent (7). Each of these reagents has its advantages and disadvantages. The former is much less sensitive than the latter. The latter will show bright blue fluoresence with compounds such as gitoxin and other substances in concentrations that are not readily detected by the Raymond reagent. Furthermore a blue fluorescing substance may have the same R_f value as some other glycoside and thus completely mask a yellow or some other color that might be present. I have shown the presence of some substances in some of the species of digitalis that give a red color in the sulfuric acid layer in the Keller-Kiliani color reaction that do not have digitoxose and do not give a Raymond reaction on paper but do give a strong blue fluoresence with the trichloroacetic acid reagent. These substances apparently are not saponins.

Extracts from Brief Enzymatic Treatment.—Approximately 600 to 800-Gm. lots of the fresh leaves of each of the following digitalis species; *purpurea*, *siberica*, *ferruginea*, *mertonensis*, and *amandiana* were collected in the fall of 1956. These were treated as previously described using approximately 15% aqueous methanol as the solvent to obtain the primary extracts. Each of these extracts was extracted with three successive portions of a mixture of ether and methylene chloride (in a ratio of 3 to 1) and labeled extract *A*. This was followed by three successive extractions with ethyl acetate which when combined was labeled extract *B*. The above extracts were examined by paper chromatography.

The results of the studies recorded in this paper are directed toward the identification of those digitalis glycosides that occur in major amounts. Figure 1 is a typical chromatogram that was obtained from extracts *A* with solvent system I and the Raymond reagent. Figure 2 is a typical chromatogram that was obtained from extracts *A* with solvent system I and the trichloroacetic acid reagent. These two figures in part, substantiate the preceding discussion. This can be further illustrated by Fig. 3 in which special care was taken to place the same amounts at the starting line. The chromatogram before spraying with the reagents was cut longitudinally along the dotted lines indicated in Fig. 3.

Extracts *B* when examined paper chromatographically using solvent systems I and II showed the absence of the desglucoglycosides. Some of these extracts showed the presence of glycosides that moved on paper in the proximity of lanatoside C. No further extensive studies were carried out on these extracts at this time.

Isolation of the Crystalline Glycosides.—The following general procedure was used. The solid material that was obtained from extract *A* was dissolved in about 20 cc. of methylene dichloride and then diluted with 60 cc. of ether. This mixture was extracted quickly with two to four successive small portions of 1% aqueous sodium hydroxide. The organic layer was then washed once with 1% aqueous acetic acid and lastly once with water. At this point, in some cases, glycosidic material separated rather quickly and was subsequently identified as gitoxin, acetylgitoxin, or unknown glycosidic material depending on the species of plant under examination. The solvent was removed and the residue digested several times with petroleum ether.

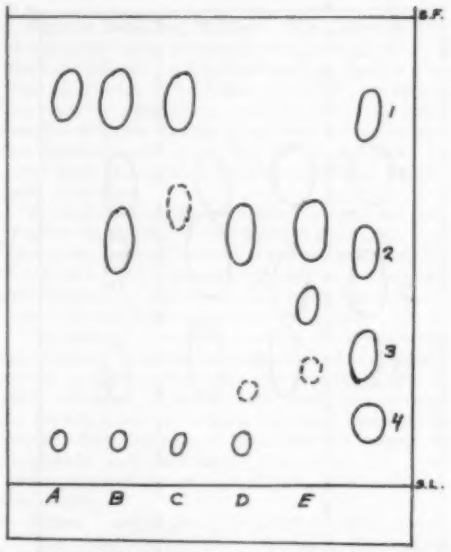

Fig. 1.—Solvent system I; S.F. = solvent front; S.L. = starting line; 1 = acetyldigitoxin; 2 = digitoxin; 3 = acetyldigoxin; 4 = digoxin; A = *Digitalis ferruginea;* B = *Digitalis amandiana;* C = *Digitalis siberica;* D = *Digitalis mertonensis;* E = *Digitalis purpurea.* Raymond reagent used to locate the glycosides.

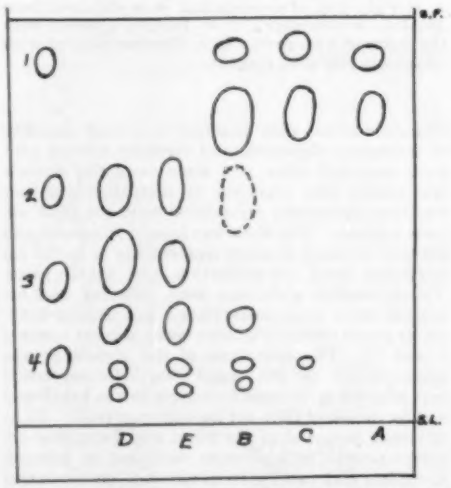

Fig. 2.—Same as that for Fig. 1 except trichloroacetic acid was used to locate the glycosides.

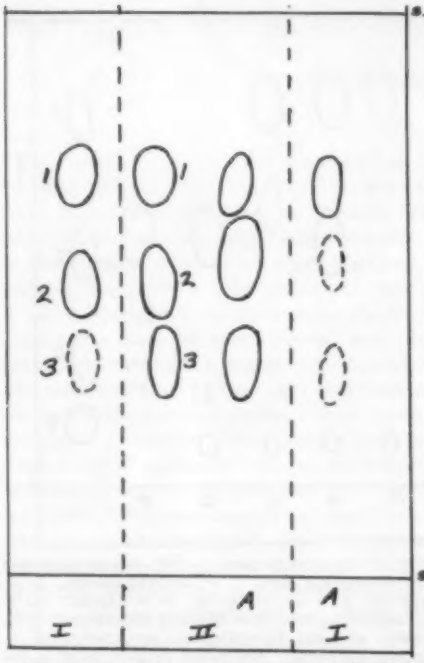

Fig. 3.—Solvent system 1; S.F. = solvent front; S.L. = starting line. 1 = Digitoxin; 2 = acetyldigoxin; 3 = gitoxin (as an impurity in digitoxin). A = First crop of crystals that were obtained from *Digitalis mertonensis*. I = Portion sprayed with the Raymond reagents; II = Portion sprayed with trichloroacetic acid reagent.

The residue was then dissolved in a small quantity of methylene dichloride and carefully diluted with pure isopropyl ether. In some cases the mixture was seeded with digitoxin or acetyldigitoxin that had been previously crystallized from the same solvent mixture. The flask was loosely stoppered and allowed to stand at room temperature or in the refrigerator until crystallization had taken place. The crystalline glycosides were collected and examined *per se*, after deacetylation and after hydrolysis by paper chromatography using solvent systems I and II. The hydrolysis of the glycosides was accomplished in the usual way. Deacetylation was effected by sodium hydroxide in the usual way and by the use of IRA-401 ion exchange resin. IRA-401 resin prepared in the usual way gave slow deacetylation in 85% aqueous methanol or ethanol. Complete and rapid (fifteen minutes) deacetylation was accomplished when the base form of the IRA-401 resin was used in a finely powdered form.

The results of the above studies showed that the crystalline glycosides were digitoxin and gitoxin from *Digitalis mertonensis* and *Digitalis purpurea*, and acetyldigitoxin from *Digitalis siberica*, *Digitalis ferruginea*, and *Digitalis amandiana*.

Figure 4 represents a typical chromatogram obtained in the characterization of these crystalline glycosides.

Figure 5 illustrates the use of system II for the

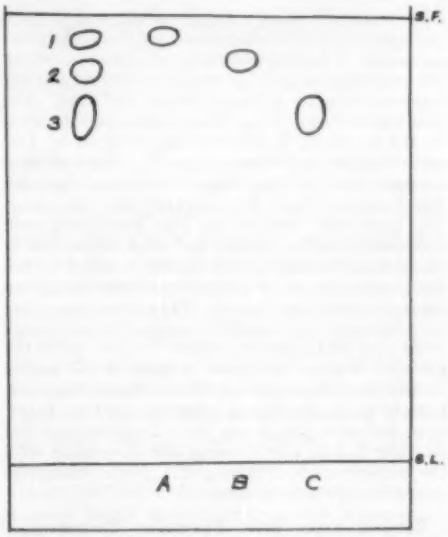

Fig. 4.—Solvent system I; S.F. = solvent front; S.L. = starting line; 1 = Acetyldigitoxin; 2 = digitoxigenin; 3 = digitoxin. A = Crystalline glycoside from *Digitalis siberica;* B = A hydrolyzed; C = A deacetylated. Raymond reagent used to locate the glycosides and aglycons.

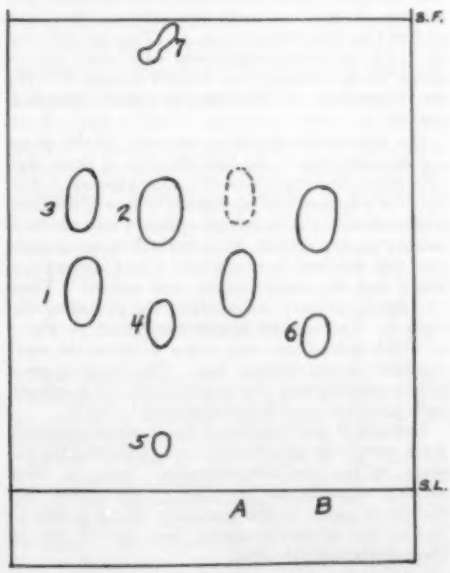

Fig. 5.—Solvent system II; S.F. = solvent front; S.L. = starting line. 1 = gitoxin; 2 = gitoxigenin from hydrolyzed gitoxin; 3 = impurity in gitoxin (gitoxigenin); 4, 5, and 6 = brown spots resulting from the hydrolysis of gitoxin and gitoxin from *Digitalis mertonensis;* 7 = anhydrogitoxigenin. A = gitoxin from *Digitalis mertonensis;* B = A hydrolyzed. Trichloroacetic acid reagent used to locate the glycosides and aglycons.

characterization of the gitoxin that was obtained from *Digitalis mertonensis*. A blue spot was obtained for gitoxin, the gitoxin from *Digitalis mertonensis* and the first crop of crystalline material (See Fig. 3) from *Digitalis mertonensis* that has the same position on the paper as gitoxigenin. In some cases a bright blue spot followed the solvent front after the hydrolysis of the gitoxins. This possibly could be an anhydrogitoxigenin.

Figure 6 shows how excellent separation can be obtained between digitoxin and digitoxigenin at a temperature of 24° or slightly lower.

Digitoxin from Digitalis Mertonensis.—When extract *A* from 600 Gm. of fresh leaves of *Digitalis mertonensis* was concentrated to a small volume and allowed to evaporate slowly to dryness some of the glycosides crystallized. The mass thus obtained was carefully digested with methylisobutyl ketone or a mixture of methylene dichloride and ether (1:3). A clean separation of white crystalline glycosides was obtained that weighed 300 mg. The above results were reproducible. Digestion with methylene dichloride left a residue that was chiefly gitoxin. One recrystallization of the methylene dichloride soluble fraction from a mixture of methylene dichloride and ether gave 165 mg. of digitoxin that melted on the Koefler Block at 221–223°. A second crop of 61 mg. of digitoxin was obtained.

The isolation of digitoxin from *Digitalis mertonensis* by the methods described in this paper appear to be both direct and simple. When this method was applied to *Digitalis purpurea* no direct separation of digitoxin was obtained from the initial extract *A*. Digitoxin was obtained in a crystalline state only when extract *A* was processed under the general procedure.

Enzyme Inhibiting Studies.—The following general procedure was adopted to examine the nature of the native digitalis glycosides found in the following digitalis species, i. e., *lanata, purpurea, ferruginea, mertonensis, amandiana,* and *siberica*. A small handful of leaves was placed in about 125 cc. of boiling distilled water and boiled for five minutes. In some cases the distilled water was buffered at pH 7 with phosphate.

A phosphate buffer was used to prevent any possible hydrolysis of the digitalis glycosides. Although no great differences between the use of distilled water and a phosphate buffer were observed, nevertheless, it appeared that better results were obtained when a phosphate buffer was used.

The mixture then was blended for five minutes and filtered. The filtrate was extracted as described under the general procedure and extracts A_1 and B_1 were obtained. These extracts were concentrated to dryness under a vacuum and then dissolved in about a 2-cc. mixture of equal parts of methylene dichloride and methanol. These extracts were examined paper chromatographically with the following results.

Figures 7 and 8 clearly demonstrate the absence of the desglucoglycosides. Extracts A_1 contain chiefly the A series of Stoll's native glycosides whereas extracts B_1 contain the C series and/or other glycosidic substances. No attempt has been made at this time to resolve the minor glycosides found in the digitalis species investigated. Paper chromatograms clearly show that minor glycosides are present some of which may not be affected by the short

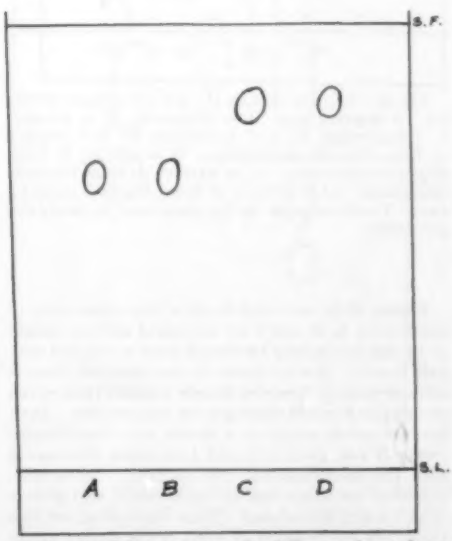

Fig. 6.—Solvent system I; S.F. = solvent front; S.L. = starting line. A = Digitoxin; B = crystalline glycoside from *Digitalis mertonensis;* C = A hydrolyzed; D = B hydrolyzed. Raymond reagent used to locate the glycosides and aglycons.

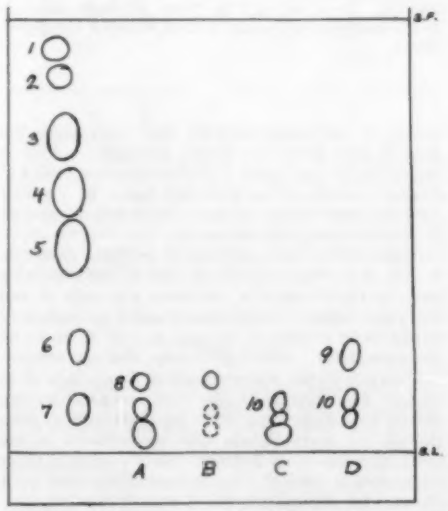

Fig. 7.—Solvent system II; S.F. = solvent front; S.L. = starting line. 1 = Acetyldigitoxin; 2 = digitoxin; 3 = acetyldigoxin; 4 = gitoxin; 5 = digoxin; 6 = lanatoside A; 7 = lanatoside C. A = extract B_1 from *Digitalis purpurea;* B = extract A_1 from *Digitalis purpurea;* C = extract B_1 from *Digitalis lanata;* D = extract A_1 from *Digitalis lanata.* Trichloroacetic acid reagent used to locate the glycosides.

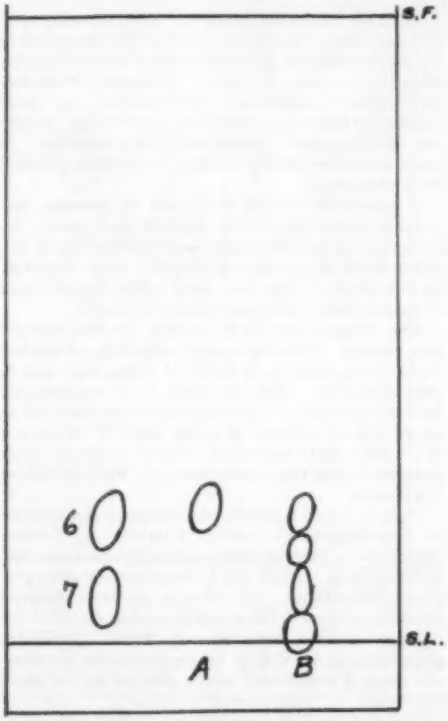

Fig. 8.—Solvent system II. 6 = Lanatoside A; 7 = lanatoside C. A = extract A_1 from *Digitalis siberica;* B = extract B_1 from *Digitalis siberica.* Trichloroacetic acid reagent used to locate the glycosides.

period of enzymatic activity that hydrolyzes the glucose unit from the major glycosides. This is probably the case with *digitalinum verum* found in *Digitalis lanata* and a glycoside found in *Digitalis purpurea* that moves on paper with solvent system II the same as *digitalinum verum.*

Figure 7 shows the presence of purpurea glycoside A (No. 8) in both extracts A_1 and B_1, the remaining spots possibly could be purpurea glycoside B and other glycosides. Lanatosides A and C are definitely shown to be present in extracts A_1 and B_1 as 9 and 10 respectively. Other glycosides also are present.

Figure 8 shows the presence of lanatoside A in extract A_1. Extract B_1 also contains some lanatoside A and what appears to be lanatoside C even though no acetyldigoxin was encountered in the extracts made from *Digitalis siberica* after a short fermentation period. An intense deep blue spot remained at the starting line in extract B_1.

In Fig. 9, extract A_1 from *Digitalis mertonensis* shows the pronounced spots comparable to lanatoside A and B. This was quite unexpected because no acetyldigitoxin or acetylgitoxin could be detected in extracts that were made from this plant following a short fermentation period. Lanatoside C appeared to be absent whereas the presence of lanatoside B is indicated. A descending paper chromatogram was run for sixteen hours to verify the absence of lanatoside C and to confirm the presence of lanatoside B. Extract B_1 from *Digitalis mertonensis* gave a chromatographic picture the same as extract A_1, however the spot for lanatoside A was weaker and the spot for lanatoside B was much brighter.

The chromatographic results of extract A_1 from *Digitalis amandiana* was strikingly different from those obtained from the other species in that a very pronounced spot was obtained that indicated the presence of gitoxin in addition to one for lanatoside A. The presence of lanatoside A also was shown in extract B together with what appeared to be lanatoside B.

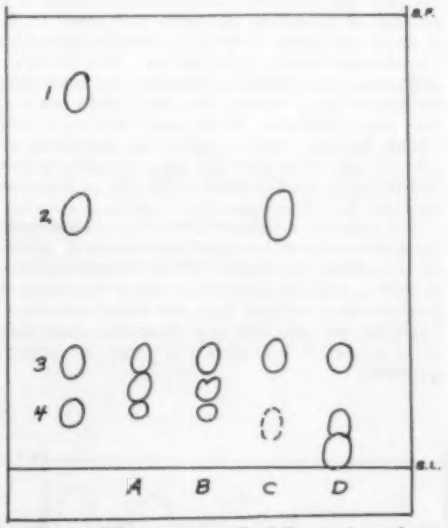

Fig. 9.—Solvent system II; S.F. = solvent front; S.L. = starting line. 1 = Digitoxin; 2 = gitoxin; 3 = lanatoside A; 4 = lanatoside B. A = extract A_1 from *Digitalis mertonensis;* B = extract B_1 from *Digitalis mertonensis;* C = extract A_1 from *Digitalis amandiana;* D = extract B_1 from *Digitalis amandiana.* Trichloroacetic acid reagent used to locate the glycosides.

Figure 10 is recorded to show the separation of lanatosides A, B, and C by the use of solvent system II by the descending technique after seven and one-half hours. Shorter times do not separate lanatosides B and C, however longer periods than seven and one-half hours effect greater separations. *Digitalinum verum* moves at a slower rate than lanatoside B and gives a bright blue-white fluoresence with the trichloroacetic acid reagent. Strospeside moves at the same rate as lanatoside A and gives a bright white fluoresence. This chromatogram also shows the composition of some of the native glycosides of *Digitalis lanata* (enzyme inhibited extraction).

Chromatographic Analysis of Gitalin.—Gitalin when examined paper chromatographically (see

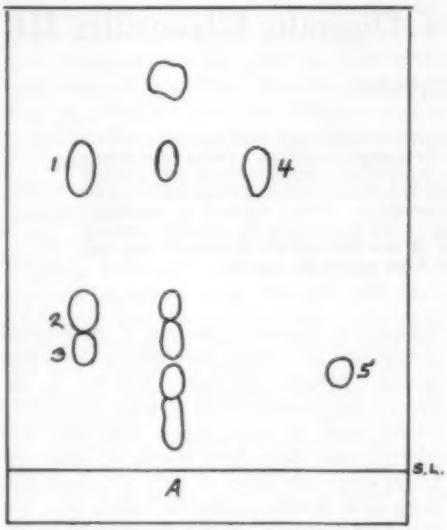

Fig. 10.—Solvent system II; descending technique; S.L. = starting line. 1 = Lanatoside A; 2 = lanatoside B; 3 = lanatoside C; 4 = strospeside; 5 = *digitalinum verum;* A = extract prepared from enzyme inhibited *Digitalis lanata*. Trichloroacetic acid used to locate the glycosides.

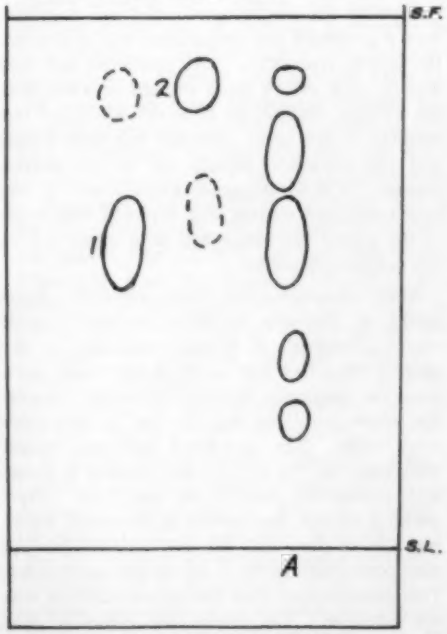

Fig. 11.—Solvent system II; S.L. = starting line; S.F. = solvent front. 1 = Gitoxin; 2 = digitoxin; A = gitalin. Trichloroacetic acid reagent used to locate the glycosides.

Fig. 11) using solvent system II showed the presence of five distinct spots one of which corresponded to gitoxin and another to digitoxin. The remaining spots gave a blue fluoresence under ultraviolet light.

Studies with Dried Digitalis Purpurea.—Some fresh leaves of *Digitalis purpurea* were dried in an incubator at 60° overnight. The dried leaves were pulverized and extracts made as previously described. These extracts when examined by solvent system II definitely indicated that considerable digitoxin and gitoxin had been generated by enzymatic activity during the drying of the leaves, however, it was by no means complete.

The results of these preliminary studies are recorded in Fig. 12.

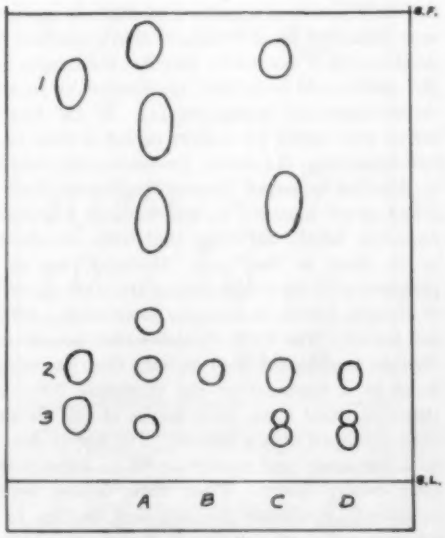

Fig. 12.—Solvent system II; S.F. = solvent front; S.L. = starting line. 1 = digitoxin; 2 = lanatoside A; 3 = lanatoside C. A = extract *A* from *Digitalis purpurea* dry leaves; B = extract *B* from *Digitalis purpurea* dry leaves; C = extract A_1 from *Digitalis purpurea* dry leaves; D = extract B_1 from *Digitalis purpurea* dry leaves.

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Enzymatic Decomposition of Digitalis Glycosides III*

By OLE GISVOLD

Enzyme inhibiting and enzyme favoring extraction techniques were applied to dried *Digitalis purpurea* leaves and to Digitalis Reference Standard. Paper chromatographic examination of the various extracts showed that digitalis leaves when dried carefully below 60° and Digitalis Reference Standard contained large amounts of the native glycosides such as purpurea glycoside-A. When ethanol or methanol concentrations of 66 per cent or greater were used to prepare the primary extracts enzymatic inhibition was obtained. A pH of one favored the enzymatic removal of the terminal glucose unit of the native glycosides.

PRELIMINARY phytochemical investigations from this laboratory have shown that when the fresh leaves of a number of digitalis species were extracted by a technique that permitted a short period of enzymatic activity, the desglucoglycosides could be isolated or detected by paper chromatographic techniques (1). If the fresh leaves were boiled for a short period of time before extraction, the native glycosides only could be detected by paper chromatographic methods. I had never intended to reinvestigate *Digitalis purpurea*, falsely believing that little remained to be done in this area. However, my experiences with the fresh leaves of the other species of digitalis led me to instigate some studies with dry leaves. The U. S. P. states that powdered digitalis is prepared from digitalis that has been dried at a temperature not exceeding 60°. I therefore dried some fresh leaves at 60° in an oven equipped with a blower. The leaves dried in a few hours and readily could be pulverized after twelve hours. When these leaves were examined by enzyme favoring and enzyme inhibiting techniques previously described (1) the results were somewhat comparable to those obtained from the fresh leaves. Some of the desglucoglycosides were present in the dried leaves and much of the native glycosides were also present. Dried leaves of *Digitalis purpurea* obtained from Callison Co. also were examined according to the same techniques and similar results were obtained. In this case it appeared that less desglucoglycosides were present in the dried leaf. An extract of the dried leaves made with 40 per cent (or less) methanol did not inhibit the enzyme that cleaved the terminal glucose residue from the native glycosides whereas 66 per cent (or stronger) methanol or ethanol completely inhibited the enzymatic activity.

When the fresh leaves of *Digitalis purpurea* were dried at 100° and subsequently examined by enzyme inhibiting techniques, only the desglucoglycosides could be detected. This implied that enzymatic cleavage of the terminal glucose unit had taken place during the drying process. Because enzymatic activity is destroyed by plunging fresh or dried leaves into boiling water, it would appear that enzymatic activity must have been completed while the leaves were warming up to 100°. These studies being of a strictly qualitative nature were not able to detect any minute decompositions enzymatic or otherwise that may have taken place beyond the digitoxin stage.

An alcoholic (enzyme inhibiting concentrations) extract from powdered *Digitalis purpurea* containing the desglucoglycosides was prepared from a powdered leaf preparation that contained the native glycosides. The powdered leaf was first covered with a small amount of water and the mixture digested at 40 to 60° for about ten minutes to one hour. Alcohol was then added and the extraction carried out in the desired manner. Chromatographic examination of extracts prepared in this way revealed that most of the native glycosides had been converted to the desglucoglycosides.

After demonstrating that carefully dried leaves of *Digitalis purpurea* contained much native glycosides, it became necessary to determine the effect that a pH of one would have upon the enzymatic activity of the leaf because the whole powdered digitalis leaf is sometimes used orally. The powdered leaf was mixed with water having a pH 1 and allowed to stand with occasional shaking for one hour. After working up this preparation in the usual way I was able to show that the native glycosides had been converted chiefly to the desglucoglycosides. This demonstrated that the enzyme activity was not inhibited. The results also indicated that under the conditions of the experiment only removal of the terminal glucose residue had taken place.

Strospeside occurs in the extracts obtained from both the enzyme inhibiting and enzyme favoring procedures.

* Received April 25, 1958, from the College of Pharmacy, University of Minnesota.

Enzyme inhibiting and enzyme favoring techniques when applied to U. S. P. Digitalis Reference Standard 1942 also gave the same results that were obtained from the dried leaves. It can be concluded that this reference powder contained the active cardiac glycosides chiefly as the native glycosides. It also implied that the enzyme that cleaves the terminal glucose residue was still active.

The above results indicate that when U. S. P. Digitalis Reference Standard 1942 was and is used for biological assay purposes, the native glycosides are the chief reference glycosides. This is because the primary extract to be used for assay purposes is made with an alcohol concentration that completely inhibits the enzymes present. In the case of the digitalis leaf or powder to be assayed much would depend on where the leaf was grown and how it was dried and stored as to the nature and amounts of the glycosides.

There is a marked difference in the MLD μg./Kg. in cats of purpurea glycoside-A which is 469 and digitoxin which is 330. The same is true for purpurea glycoside-B which is 548 and gitoxin which is 400. This is only part of the problem in the case of *Digitalis purpurea*. All prior biological assays of *Digitalis purpurea* are meaningless in terms of relating such values to oral activity in man. Although *Digitalis purpurea* may contain variable amounts (0 to very high percentages) of purpurea glycoside-B (or gitoxin) these glycosides are not absorbed orally in a cat and this also is very probably true in humans. Strospeside also occurs in variable amounts in *Digitalis purpurea* and even though it has a MLD μg./Kg. in cats of 586, its physical properties probably preclude it from being absorbed orally. Nothing is known of the oral absorption of purpurea glycoside-A but it is probably considerably lower than digitoxin. Digitoxin on the other hand is claimed to be excellently absorbed if not 100 per cent.

It would appear therefore that an assay is needed to determine the oral potency of *Digitalis purpurea*. This might be done in animals if the results could be correlated in humans. An alternate solution would be an assay involving a determination of the amounts of the orally absorbable glycoside or glycosides by physical, chemical, or instrumental methods. In this case an assay for the digitoxin content probably would be most useful.

Prior assays (colorimetric) for the glycoside content of *Digitalis purpurea* also had little significance. This is because such assays were based upon the presence of the α,β-unsaturated lactone ring which is present in all the cardioactive glycosides whether they are orally absorbed or not. Such assays suffered from the same disadvantages as did the biological assays.

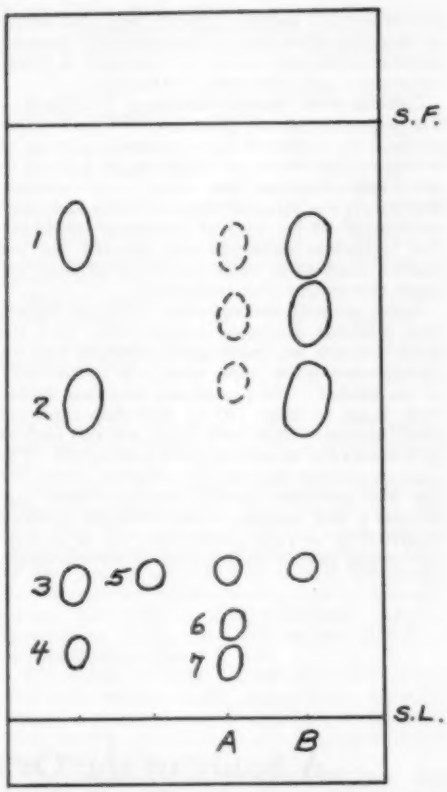

Fig. 1.—Solvent system II; S. F. = solvent front, S. L. = starting line. 1 = digitoxin; 2 = gitoxin; 3 = lanatoside-A; 4 = lanatoside-C; 5 = strospeside; 6 = purpurea glycoside-A; 7 = purpurea glycoside-B. A—Represents the chromatogram of an extract obtained from *Digitalis purpurea* powder dried at 60° or less or U. S. P. Digitalis Reference Standard. The extracts were prepared by the enzyme inhibiting techniques, i. e., boiling water or strong alcohol. B—Represents the chromatogram of an extract obtained from *Digitalis purpurea* powder dried at 60° or less or U. S. P. Digitalis Reference Standard. The extracts were prepared from the powdered leaf by a brief enzymatic treatment; one hour exposure to an aqueous medium at pH 1; alcohol concentrations of 40% or less or digestion with a small amount of water at 40 to 60° followed by ethanol. B—Also represents the chromatogram of an extract obtained by enzyme inhibiting techniques applied to digitalis leaf that had been dried at 100°.

EXPERIMENTAL

The details of the paper chromatographic techniques used in these studies have been described

previously (1). Solvent system II was used for the development of the paper chromatograms. Jensen's trichloroacetic acid reagent (2) was used to detect the position of the glycosides on the paper.

Extracts from Enzyme Inhibiting Treatment.—The following general procedure was used. Five grams of the powdered leaf was added to 100 cc. of boiling distilled water (or distilled water buffered to pH 7 with phosphate) and boiled for five minutes. The filtrate was then extracted with three successive portions of 30 cc. each of methylene dichloride. The methylene dichloride was removed and the residue dissolved in about two cc. of methanol for paper chromatographic examination.

When alcoholic concentrations (66% or higher) that inhibited enzymatic activity were used the drug (5 Gm.) was macerated overnight and the filtrate concentrated under vacuum to remove most of the alcohol. The concentrate was then diluted with water to make 100 cc. and then extracted three successive times with 25-cc. portions each of petroleum ether to remove much chlorophyll. The aqueous solution was then processed as above. If the final methanol solution was too highly pigmented it was partially depigmented by methods described in previous publications (1) or directly by treating it with small successive portions of the finely powdered base form of the ion exchange resin IRA-401.

Extracts from Brief Enzymatic Treatment.—Five grams of the powdered leaf was added to 100 cc. of water and the mixture warmed to 50 to 60° for ten minutes. The filtrate then was treated as described under extracts from enzyme inhibiting treatment.

Extracts from 40% Methanol.—Five grams of powdered leaf was macerated overnight with 40% methanol and the filtrate processed as described where alcoholic concentrations of 66% or higher were used to make the initial extract.

Studies comparable to those described in the above experimental part have been initiated with *Digitalis mertonensis*. These will be completed and reported at a later date.

Figure 1 has been made by reconstructing a number of actual chromatograms in order to conserve space in THIS JOURNAL. The representations are so close to the actual chromatograms that little if any accuracy has been sacrificed.

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A Study of the Oxidative Metabolism of Acetylsalicylic, Salicylic, and Gentisic Acid in Fevered Animals*

By ROSS E. CRABTREE,† JOHN B. DATA, and JOHN E. CHRISTIAN

Labeled compounds were used to study the metabolism of salicylates in rats. A method, electrophoretic separation, was developed to identify the oxidative metabolites. Analysis of tissue extracts by this method generally revealed only the administered compounds. However, gentisic acid was found in the pituitary region of fevered rats administered either acetylsalicylic or salicylic acid. It was not found in the pituitary after administration of these compounds to nonfevered rats or after administration of gentisic acid to fevered rats.

SEVERAL DRUGS form active metabolites, and in some cases these metabolites are actually responsible for the activity of the compounds. Salicylates are known to form at least one active metabolite, gentisic acid, which is found in the urine of subjects administered salicylates (1). This compound, 2,5-dihydroxybenzoic acid, has been shown (2, 3) to produce the same antipyretic effects as salicylates without the untoward effects of "salicylism." However, authorities (1) state that it cannot be the active form of salicylate, because it must be given in the same high dosage as sodium salicylate in order to obtain the same therapeutic effects. It is also stated (4) that the amount formed in the body after the administration of sodium salicylate is not sufficient to exert the action.

Recent work (5, 6) has shown that there is selective accumulation of radioactivity in certain tissues of the body after administration of labeled salicylates. No work has been reported on the form in which the radioactive material is

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present in these tissues. This study was undertaken to determine whether any metabolic products of salicylates are present in the tissues during the time in which the antipyretic action is being exerted.

EXPERIMENTAL

Synthesis of Possible Metabolites.—Gentisic acid was purchased commercially. It was electrophoretically pure and melted at 201–202° (corr.). 2-Acetylgentisic acid was prepared by the method of Bergmann and Dangschat (7). It melted at 167–168° (corr.). It proved to contain gentisic acid as an impurity when examined by electrophoresis. 2,3,5-Trihydroxybenzoic acid was synthesized by the method of Schock and Tabern (8, 9). It melted at 127–128° (corr.). It also proved to contain traces of gentisic acid when examined electrophoretically. Although gentisic acid was an impurity in both of these products, the compounds were sufficiently pure to establish their metabolic behavior.

Synthesis of Labeled Compounds.—C-14 Carboxyl salicylic and C-14 carboxyl acetylsalicylic acids were synthesized in these laboratories by Borst and Christian (10).

C-14 Carboxyl gentisic acid was synthesized from radioactive salicylic acid by the method of Brodie, *et al.* (11), using modifications necessary for the radioactive synthesis. To 250 ml. of 0.1 *M* phosphate solution, pH 6.6, there was added 207 mg. of C-14 salicylic acid, 87 mg. of $FeSO_4 \cdot 7H_2O$, 474 mg. of ethylenediamine tetraacetic acid (EDTA), and 616 mg. of ascorbic acid. The solution was kept at 36° for four hours during which time air was vigorously bubbled into the solution. After acidification with hydrochloric acid the solution was extracted 4 times with 30 ml. of chloroform followed by 5 extractions with 30 ml. of ether. On evaporation the chloroform extract yielded 90 mg. of unreacted salicylic acid. The other extract contained the gentisic acid, crude yield 60%.

In order to prove the identity of the compound isolated, a portion of the product was subjected to separation on a chromatographic column according to the general method described by Trenner (12). The solvent pair was glycerol-ethyl acetate and the holding solid was Supercel. The progress of the material on the column was followed by its fluorescence under ultraviolet light. The flourescent material was eluted from the column, and after evaporation of the solvent, white crystals remained. The product was electrophoretically pure, radiochemically pure, and it had the same melting point as gentisic acid obtained commercially. The specific activity determined in the Actigraph apparatus[1] was 740 c. p. m./mg.

Procedure.—Fever was produced in the animals by use of peptone solution according to the procedure given by Buller, *et al.* (13). The drugs were dissolved in 1 ml. of 0.1 *M* phosphate solution, pH 7.4, and administered by subcutaneous injection. Table I shows the dosage schedule in which the various compounds were administered. Forty-five minutes after injection the rats were decapitated, and the blood and urine collected. The tissues were dissected, frozen on dry ice, and stored until use.

TABLE I.—DOSAGE SCHEDULE FOR SALICYLATE COMPOUNDS ADMINISTERED TO RATS

Compounds	Dose, mg./Kg.	No. of Rats in Group	Condition
C-14 Acetylsalicylic acid	100	5	Febrile
Acetylsalicylic acid	100	10[a]	Febrile
C-14 Acetylsalicylic acid	100	2	Nonfebrile
Acetylsalicylic acid	100	10[a]	Nonfebrile
C-14 Salicylic acid	200	5	Febrile
C-14 Gentisic acid	200	6	Febrile
2-Acetylgentisic acid	150	3	Febrile
Gentisic and 2,3,5-trihydroxybenzoic acid mixture	100	3	Febrile

[a] Only the blood, urine, and pituitary were examined in these experiments.

The blood was collected over sodium citrate and immediately centrifuged. The plasma and red cells were then separated and an equal volume of acetone added to each of these fractions. Each of the plasma samples was treated separately, but the red cell extracts were pooled for measurement. The mixtures were centrifuged and the supernatant liquids dried over sulfuric acid in a vacuum desiccator at 36°.

The rest of the tissue samples were also pooled. The liver, kidneys, heart, spleen, lungs, adrenals, brain, and pituitary[2] were all separately homogenized with alcohol, centrifuged, and the supernatants evaporated *in vacuo* at 36°.

Electrophoresis.—All the compounds were subjected to electrophoresis on the Reco apparatus.[3] The separations were made on Schleicher and Schuell 2045a filter paper strips. Citrate buffer was used, pH 4.0, ionic strength 0.15. The compounds were dissolved in 1:1 ethanol/buffer solution and 0.02 ml. was applied to the negative side of the strips. Electrophoresis was run for five hours at 350 v. which gave a current of approximately 10 ma.

The spots were detected on the strips by examination under ultraviolet light, by reaction with ferric nitrate solution, and by radioactivity. The strips were scanned for radioactivity by the use of the Actigraph apparatus. The slits furnished with the instrument were replaced with a lead disk containing a 0.5-cm. slit which was positioned 1 mm. above the paper strips.

RESULTS

Various salicylate derivatives can be distinctly separated by electrophoresis. Table II gives the relative movement of the various compounds. Salicylic acid was arbitrarily assigned a R_f value of 1.0 and the movement of the compounds compared to it.

[1] Model No. C-100-Nuclear Instrument and Chemical Corp., Chicago, Ill., 1.4 mg./cm.^{2} mica window.

[2] In the case of the pituitary a section of the surrounding brain tissue on the floor of the third ventricle was also included.

[3] Model No. E-800-2, Research Equipment Corp., Oakland, Calif.

TABLE II.—ELECTROPHORETIC SEPARATION OF SALICYLATE DERIVATIVES

Compounds	R_f Value
Salicylic acid	1.00
Gentisic acid	0.80
Acetylsalicylic acid	0.70
2-Acetylgentisic acid	0.62
2,3,5-Trihydroxybenzoic acid	0.58

Acetylsalicylic Acid.—Table III shows the compounds detected in the various tissues of the fevered rats administered C-14 acetylsalicylic acid. A rough estimate of the relative concentrations of the compounds was made by comparison of the areas under the radioactivity curves. In most cases the curves overlapped so that definite quantitative values could not be assigned.

to two nonfevered rats. The findings were generally the same as those with fevered rats except that gentisic acid was not found in the pituitary extracts. Confirmatory experiments with nonradioactive acetylsalicyclic acid also failed to show any fluorescent spot in the pituitary strips for nonfevered rats.

Salicylic Acid.—The results of the administration of C-14 salicylic acid were limited by the low specific activity of the compound. Only qualitative results were recorded. Salicylic acid was the only radioactive compound found in the plasma, red blood cells, heart, lungs, spleen, liver, and brain. Gentisic acid was again detected in the kidneys, urine, and pituitary extracts. An unidentified compound was found in the adrenal extract with the same R_f value, 0.15, as found in the previous experiments.

TABLE III.—COMPOUNDS FOUND IN THE TISSUES AFTER ADMINISTRATION IF C-14 CARBOXYL ACETYLSALICYLIC ACID TO FEVERED RATS

Tissue	Acetylsalicylic Acid	Salicylic Acid	Gentisic Acid	Unknown (R_f Value)
Plasma	++++[a]	+	..	...
Red blood cells	++	++	..	...
Kidneys	..	+++	+	...
Urine	..	++++	++	+(0.19)
				++(0.57)
Heart	+	+++	..	...
Lungs	+	+	..	...
Spleen	+	++	..	...
Liver	++	+++	..	...
Brain	+	+	..	...
Pituitary	+	+	+	...
Adrenals	+	+	..	+(0.15)

[a] The concentrations are graded from + to ++++ according to the relative areas under the radioactivity curves.

In general, only acetylsalicylic and salicylic acids were found in the tissues. However, certain exceptions are noteworthy. Gentisic acid was found in the kidneys, urine, and pituitary extracts. Two unidentified products were detected in the urine. One unidentified product was also found in the adrenal extract.

The three radioactive spots on the pituitary strip were sufficiently separated so that they could be cut out and the radioactivity determined under a Geiger-Müller tube. The three sections corresponding to salicylic, gentisic, and acetylsalicylic acids gave equal activity demonstrating that gentisic acid was present in the pituitary in approximately equal concentration to the amount of salicylic acid and to the amount of acetylsalicylic acid. The section corresponding to gentisic acid was eluted with alcohol and pure gentisic acid was added. Electrophoresis of this solution gave only one spot. The spot was fluorescent and contained all the radioactivity. This was taken as proof that the compound in the pituitary was gentisic acid.

For confirmation of these findings two groups of five fevered rats were administered actylsalicylic acid. In both groups the florescent spot corresponding to gentisic acid appeared on the pituitary strips. Gentisic acid was also found in the urine but not in the plasma.

C-14 Acetylsalicylic acid was also administered

Gentisic Acid.—The labeled gentisic acid was also of low specific activity and only qualitative results were recorded. Gentisic acid was found in most of the tissue examined. A radioactive peak corresponded to the bright blue fluorescent spot of gentisic acid in every case except the pituitary. In the pituitary a radioactive spot, R_f value 0.73,[4] was found, but no fluorescence was observed. In a number of other tissues radioactivity was also recorded in a nonfluorescent section with a R_f value of approximately 0.70. These tissues were the plasma, red cells, liver, and adrenals. In the urine two fluorescent spots were observed to be radioactive, R_f values of 2.01 and 1.21. There was a third spot in the urine which did not fluoresce but was radioactive, R_f value of 0.63.

Apparently gentisic acid occurs in widespread body distribution. The widespread occurrence of the nonfluorescent compound indicates that gentisic acid is changed by the body into another form which does not possess fluorescent properties.

2-Acetylgentisic Acid.—Only one fluorescent spot corresponding to gentisic acid could be detected on the electropherograms after the administration of 2-acetylgentisic acid to fevered rats. Hydrolysis of the spots by placing the strips in an ammonia

[4] In these results gentisic acid was used as the comparison compound (R_f = 1.0).

atmosphere failed to reveal a second spot for 2-acetylgentisic acid. The pituitary again had no fluorescence to indicate the presence of gentisic acid. The urine patterns corresponded to those found after the administration of gentisic acid. Apparently 2-acetylgentisic acid is quickly hydrolyzed in the body.

2,3,5-Trihydroxybenzoic Acid.—Administration of the mixture of gentisic and 2,3,5-trihydroxybenzoic acids gave electrophoretic patterns which were quite similar to those of gentisic acid itself. In most of the tissues only the two administered compounds were detected. However, the plasma and red cells produced only one spot, that of gentisic acid. The trihydroxy acid could not be recovered from plasma when added *in vitro* which may explain why it could not be detected *in vivo*.

The urine strips showed a definite spot for the trihydroxy acid. This indicates that the trihydroxy compound is at least partly excreted unchanged.

DISCUSSION

Pallot and Eberhardt (6) have reported that after the administration of labeled sodium salicylate to rats the radioactivity accumulates in the anterior pituitary in a greater concentration than in any other tissue except the blood. In the present work it was shown that in febrile rats a significant part of this radioactivity in the pituitary is composed of gentisic acid. The presence of this metabolite in the pituitary is highly significant since many workers believe that one of the main actions of salicylates is activation of the hypothalamic-hypophyseal-adrenal axis (14–16). Since a high concentration of gentisic acid was found in this region, it was actually present at the site of action in sufficient quantity to exert the action. The absence of gentisic acid in the pituitary of nonfebrile rats might be one reason for the failure of salicylate to lower normal body temperature to subnormal levels.

Udenfriend, *et al.* (17), found that a mixture of ascorbic acid, EDTA, and ferrous ion will bring about hydroxylation of a number of drugs. In this connection it may be pointed out that ascorbic acid occurs in high concentration in the pituitary (18). This fact, combined with the relatively high concentration of radioactive salicylate which accumulates there, indicates that this could be one mechanism by which the gentisic acid is formed in the pituitary.

The effects which occur after the administration of acetylsalicylic acid are complex and cannot be expained solely by activation of the hypothalamic-hypophyseal-adrenal axis. The total effect arises from stimulation or depression of several systems of the body. Since widespread body distribution of any salicylate metabolite was not found, the peripheral actions of salicylate are probably due to the salicylate itself. This does not exclude the possibility that the salicylate may form complexes with certain compounds of the body which in turn may exert these actions.

Dumazert and El Ouachi (19) detected levulinic acid in the urine of rabbits and humans after the administration of sodium salicylate. They postulated that it arose from rupture of the ring of 2,3,5-trihydroxybenzoic acid during these hydrolysis procedure. In the past work it was shown that 2,3,5-trihydroxybenzoic acid is partly excreted unchanged. The trihydroxy acid spot was identified by its yellow color in visible light and its strong fluorescence under ultraviolet. 2,3,5-Trihydroxybenzoic acid probably is not a metabolite of salicylates in rats. A spot with approximately the same R_f value was also found in the urine of rats treated with acetylsalicylic acid, but the spot was not yellow in visible light and did not fluoresce under ultraviolet. Hence, the spot was not 2,3,5-trihydroxybenzoic acid. The unidentified spots found on the urine strips were probably conjugated products since hydrolysis of the urine strips in an ammonia atmosphere and separation of the alcoholic eluates gave only spots for gentisic and salicylic acids.

SUMMARY

1. A method of electrophoretic separation was developed for oxidative metabolites of salicylates.

2. Synthesis of possible salicylate metabolites is reported. These are C-14 carboxyl gentisic acid, 2-acetylgentisic acid, and 2,3,5-trihydroxybenzoic acid.

3. The metabolic products found in various rat tissues after the administration of labeled acetylsalicylic acid, salicylic acid, and gentisic acid were determined. Gentisic acid was found in the pituitary, kidneys, and urine after administration of acetylsalicylic acid and salicylic acid to fevered rats but not in any other tissues.

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The Relationships between the Histamine Liberating and Hypotensive Actions of 1-Phenyl-2-Dimethylaminoethoxymethyl Tetrazole Hydrochloride (TT-209)*

By M. B. SLOMKA

1-Phenyl-2-dimethylaminoethoxymethyl tetrazole hydrochloride (TT-209) produces a profound hypotension of long duration in the dog; refractoriness to the hypotensive action of TT-209 develops on repeated administration. TT-209's ability to produce hypotension is associated with its ability to increase plasma histamine concentrations. TT-209 and compound 48/80 demonstrate a cross-refractoriness in their ability to affect dog blood pressure and plasma histamine concentration. It is concluded that the hypotensive action of TT-209 is mediated via a mechanism of histamine liberation.

FROM AN EXTENSIVE ANALYSIS of the hypotensive action of 1-phenyl-2-dimethylaminoethoxymethyl tetrazole hydrochloride (TT-209), Yim, Gross, and Keasling suggested that this action of the compound was due to its direct depressant action on the cardiac and vascular smooth muscle (1). The similarity of the hypotensive action of TT-209 to that of known histamine liberators suggested that this action of the compound might be mediated via a mechanism of histamine liberation. This possibility was tested in the experiments reported in this communication.

EXPERIMENTAL

Methods.—Blood pressure was recorded by means of a mercury manometer from a common carotid artery of mongrel dogs anesthetized with sodium pentobarbital 33 mg./Kg. All drugs were injected rapidly as saline solutions into a femoral vein. In those experiments in which blood samples were obtained for analysis of plasma histamine the samples were withdrawn from the contralateral femoral vein from one to two minutes after drug injection into heparinized syringes.

Histamine bioassay was performed on guinea pig ileum strips suspended in an atropinized (atropine sulfate 10^{-6}) Tyrode's solution at 37°. Strips were standardized to histamine prior to each addition of dog plasma. Pharmacologic identification of histamine in plasma was established by the ability of Benadryl (10^{-7}) to inhibit plasma contractions to the same degree as histamine contractions.

Results.—The prolonged hypotensive effect of 10 mg./Kg. TT-209 in pentobarbital anesthetized dogs and the development of refractoriness to this action of the compound is illustrated in Fig. 1. The TT-209 refractory dog demonstrates a cross-refractoriness to the hypotensive action of 200 μg./Kg. compound 48/80 but not to the hypotensive action of 0.1 cc./Kg. 10% Tween 20.

The administration of 200 μg./Kg. compound 48/80 produces a hypotension similar in intensity and duration to that seen with TT-209; the animals are refractory to further administration of compound 48/80. The compound 48/80 refractory animal demonstrates a cross-refractoriness to the hypotensive action of 10 mg./Kg. TT-209.

The mean fall in blood pressure of 86% produced by 10 mg./Kg. TT-209 was associated with a mean increase in plasma histamine concentration of 0.35 μg./cc. (Table I); 200 μg./Kg. compound 48/80 produced a similar mean fall in blood pressure, 85%, and mean increase in plasma histamine concentration, 0.44 μg./cc., (Table III). In dogs made refractory to TT-209 or compound 48/80 by prior administration of either compound TT-209 or compound 48/80 produced at most transient depressor effects that were associated with no measurable increases in plasma histamine concentration. (Tables II and III).

Discussion.—The magnitude and duration of the hypotensive response and the development of tolerance to 10 mg./Kg. TT-209 agrees with that previously reported by Yim, Gross, and Keasling (1). The hypotensive activity and the development of refractoriness to such activity of compound 48/80 has been previously reported (2, 3, 4). Evidence

TABLE I.—THE EFFECT OF 10 MG./KG. TT-209 ON DOG BLOOD PRESSURE AND PLASMA HISTAMINE CONCENTRATION

Dog No.	Blood Pressure Control, mm. Hg	Fall, %	Histamine Plasma Concn., μg./ml. Control	1–2 Min. Post Drug
B	160	88	None*	0.21
E	124	81	None*	0.36
F	122	85	None*	0.36
I	144	83	None*	0.23
GG	128	87	None*	0.36
JJ	138	90	None*	0.30
KK	110	92	None*	0.32
QQ	136	90	None*	0.59
$\bar{x} \pm S_x$		86 ± 1.3		0.35 ± 0.04

* None indicates less than 0.063 μg./0.5 ml., the plasma volume used to assay for histamine.

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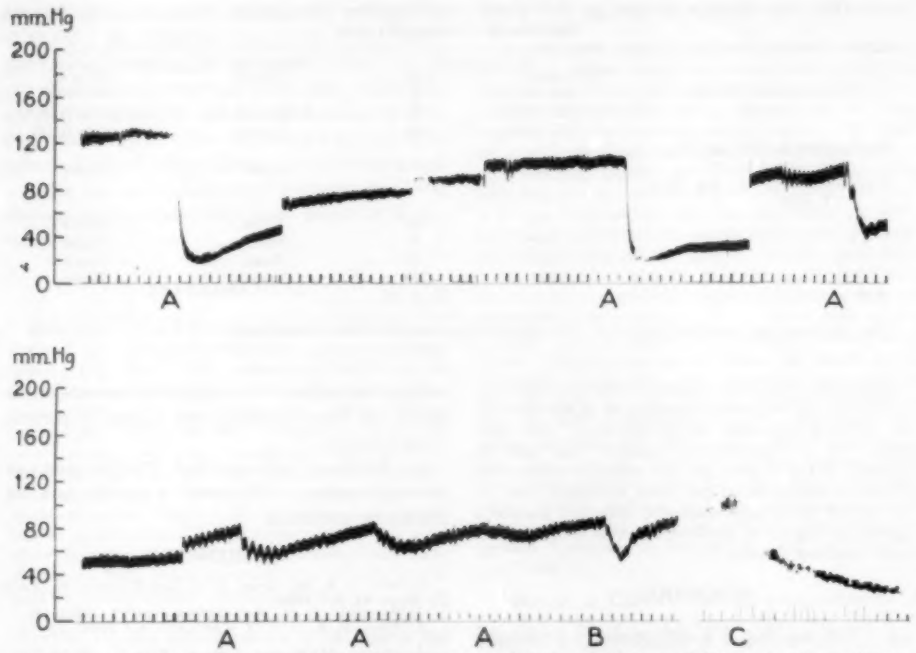

Fig. 1.—Carotid blood pressure, time marks one minute; kymograph stops of fifteen to thirty minutes. Legend:—*A* marks injection of 10 mg./Kg. TT-209, *B* marks injection of 200 μg./Kg. compound 48/80; *C* marks injection of 0.1 ml./Kg. 10% Tween 20.

TABLE II.—THE EFFECT OF 10 MG./KG. TT-209 ON DOG BLOOD PRESSURE AND PLASMA HISTAMINE CONCENTRATION AFTER PRETREATMENT

Pretreatment Dog No.	Blood Pressure Control mm. Hg	Blood Pressure Fall, %	Histamine Plasma Concn., μg./ml. Control	Histamine Plasma Concn., μg./ml. 1–2 Min. Post Drug
Refractory to 10 mg./Kg. TT-209				
E	118	17	None[a]	None[a]
F	78	0	None[a]	None[a]
GG	82	24	None[a]	None[a]
LL	104	10	None[a]	None[a]
NN	144	87	None[a]	None[a]
Refractory to 200 μg./Kg. compound 48/80				
H	140	17	None[a]	None[a]
J	106	5	None[a]	None[a]
OO	100	0	None[a]	None[a]
$\bar{x} \pm S_x$		20 ± 10		

[a] None indicates less than 0.063 μg./0.5 ml., the plasma volume used to assay for histamine.

indicates liberation of endogenous histamine to be the mechanism by which compound 48/80 exerts its hypotensive activity (2–5). The cross-refractoriness shown between TT-209 and compound 48/80 in their ability to produce their prolonged hypotensive responses would indicate that TT-209 is also producing this effect via a mechanism of endogenous histamine liberation. This mechanism of action is supported by the apparent relationships between the ability of TT-209 to produce its characteristic effect on the blood pressure and to affect plasma histamine concentration in previously untreated dogs and its inability to affect these parameters in TT-209 or compound 48/80 refractory dogs.

The inability of diphenhydramine to block the hypotensive response to TT-209 (1) is not inconsistent with the proposed hypothesis. Although antihistaminic agents can block the response to small doses of histamine, they are not effective in blocking the hypotensive response to large doses of exogenous histamine (6) or the amounts of histamine presumably released in close proximity to histaminergic receptors by histamine liberators or in anaphylaxis (7).

TABLE III.—THE EFFECT OF 200 μG./KG. COMPOUND 48/80 ON DOG BLOOD PRESSURE AND PLASMA HISTAMINE CONCENTRATION

Pretreatment Dog No.	Blood Pressure Control, mm. Hg	Blood Pressure Fall, %	Histamine Plasma Concn., μg./ml. Control	Histamine Plasma Concn., μg./ml. 1–2 Min. Post Drug
None	110–160	85	0.13 ± 0.04	0.57 ± 0.10
	110–160	85	None[a]	0.44 ± 0.05
Refractory to 200 μg./Kg. compound 48/80	80–160	0–10	None	None
Refractory to 10 mg./Kg. TT-209				
GG	78	59	None	None
LL	108	0	None	None
NN	146	0	None	None
TT	119	30	None	None
$\bar{x} \pm s_{\bar{x}}$		22 ± 14		

[a] None indicates less than 0.063 μg./0.5 ml., the plasma volume used to assay for histamine.

The ability of another known histamine liberator, Tween 20 (8), to produce prolonged hypotension in the TT-209 compound 48/80 refractory dog indicates: (*a*) a hypotensive response can still be attained in the animal, (*b*) the animal's releaseable histamine stores have not been depleted, and (*c*) TT-209 in the dosage employed does not possess a significant degree of antihistaminic activity on the dog's vascular system.

SUMMARY

1. Ten mg./Kg. TT-209 produces prolonged hypotension in the dog that is associated with an increase in plasma histamine concentration; refractoriness develops with repeated administration.

2. TT-209 and compound 48/80 demonstrate cross-refractoriness with regard to their ability to affect the blood pressure and plasma histamine concentration.

3. Evidence indicates that TT-209 produces its hypotensive action via a mechanism of histamine liberation.

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Zein as a Film-Type Coating for Medicinal Tablets*

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Zein, with and without certain additives, was added to isopropyl alcohol. Tablets coated with the resultant nonenteric fluids adequately withstand heat, abrasion, and humidity. Tastes and odors of the original tablets are concealed by the coating. Grooves and emblems on the tablet surface are retained and remain visible through the coating. The differences in solubility between the coated and uncoated tablets are negligible.

UTILIZING ESTABLISHED METHODS of sugar coating, one of the larger pharmaceutical manufacturers in this country employs more than 150 coats to cover one of their tablet products adequately. This entire process, based on a regular working schedule, requires as many as five working days. While the resultant coated tablets are pharmaceutically elegant, much remains to be accomplished with respect to decreasing the time and labor involved.

Perhaps the most expedient method of accomplishing this objective would be the development of a coating which would serve the general purposes of a nonenteric coating (1–3) without the use of subcoat powders and buildup coatings: that is, a "film-type" coating. At least one pharmaceutical manufacturer is currently producing tablets coated in this manner. Two of the more recent endeavors along these lines have been per-

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formed by Gans and Chavkin (4) and Doerr, *et al.* (5). An effort has been made to produce a nonenteric coating fluid using zein which would not require the numerous coats now employed, thereby resulting in a substantial diminution in equipment needs. Zein is resistant to microbial attack (6) and is considered to be nontoxic and suitable for use in functional coatings involving the ingestion of relatively small amounts of material (7).

EXPERIMENTAL

Materials.—The following abbreviations are used for zein and nonionic surfactants employed in this study: (Z) Zein, (T20) polyoxyethylene sorbitan monolaurate, (T80) polyoxyethylene sorbitan monooleate, (S20) sorbitan monolaurate, (S80) sorbitan monooleate, (A83) sorbitan sesquioleate, (Ax) aspirin tablets, 340 mg., Manufacturing pharmacy Laboratories, University of Illinois, (Pa) placebo tablets, 250 mg., Abbott Laboratories, 621-8914, as described by Doerr, *et al.* (5), from basic starch-lactose granulation, (Px) placebo tablets, 350 mg., Manufacturing Pharmacy Laboratories, University of Illinois, from basic starch-lactose granulation as used by Koren (8) and Villacorta (9).

Tablets (Commercially Coated).—Ammonium chloride, 7½ gr., enteric coated, Parke Davis and Company: cascara sagrada extract, 5 gr., Upjohn Company: ferrous gluconate, 5 gr., "Fergon," Winthrop Laboratories: ferrous sulfate, 3 gr., "Feosol," Smith Kline and French Laboratories: sodium salicylate, 10 gr., enteric coated, Abbott Laboratories.

Apparatus.—The apparatus used in this study included the following: 20-inch galvanized iron coating pan, 20-inch galvanized iron canvas lined polishing pan—both the coating and polishing pans were interchangeable on a motor driven bench stand and revolved at a speed of 36 r. p. m. Industrial vacuum machine (General Electric Model 203), Fisher Twin Infra-Red Rediator (each bulb rated 250 watts), U. S. P. Disintegration Device (Scientific Glass Apparatus Company, Inc.)., Shaking machine (A. S. Aloe Company), Glass Desiccator Jar, 250 mm. (Central Scientific Company), and 5X Aplanat Magnifying Lens (Bausch and Lomb).

Methods.—The coating formulas used may be explained as follows: the figures immediately following the abbreviations previously described represent the percentage by weight of the preceding material. In every instance the remainder of the formula is understood as being 91% (v/v) isopropyl alcohol, calculated on a weight basis according to the prescribed formula. For example, the formula Z-15-(T20)-3 should be read as 15% zein, 3% polyoxyethylene sorbitan monolaurate, and 82% isopropyl alcohol (91% by volume): the percentages in the formula all being on a w/w basis.

Application of Zein-Additive Fluids.—The tablets were screened, weighed, and placed in the pan ready for coating. The first application of coating fluid was added almost immediately to lessen the possibility of the uncoated tablets chipping while tumbling in the rotating pan. The fluid was applied in a thin stream along the top of the tumbling tablets. The tablets and coating were not warmed prior to application.

A maximum interval of three minutes is required between applications of the coating fluid, providing that warm air is directed onto the tablets. This time interval is dependent upon the amount of time required for the previous coating to dry. A warm air blast is necessary to shorten the drying period of each coating, thereby preventing the tablets from adhering to one another or to the sides of the pan. It is of utmost importance that this warm air (54°) be applied only after the coating has been completely distributed over all the tablets. If this procedure is not adhered to closely, the coating fluid will dry before it has covered the entire batch, resulting in an uneven coating.

When every visible tablet is evenly covered with a very thin coating of the fluid, the warm air is applied immediately. An excessive quantity of fluid produces a rough, uneven coating and leads to the possibility of tablets clumping together. The warm air treatment will usually relieve this situation, but generally when the tablets separate they are irreparably marked at the point of previous contact. Shellac sealcoat and wax polishing solutions were prepared by standard methods employing the general formulas as commonly used in industry (see Table I).

Addition of Colors.—Varying concentrations of FD & C Blue No. 1 (Brilliant Blue) and FD & C Red No. 3 (erythrosine) in Z-15-(T20)-3 were prepared. The majority of the coloring experiments were performed using the red dye since this was decided upon as being the more popular and appealing of the two colors employed.

Concentrations ranging from 0.02% to 0.5% (w/w) of FD & C Red No. 3 in Z-15-(T20)-3 were prepared. These colored zein fluids were added to the tablets in essentially the same manner as the uncolored zein fluids but failed to produce an evenly colored tablet. Initial addition of Z-15-(T20)-3 containing very small concentrations of FD & C Red No. 3, followed by subsequent coatings containing increasing dye concentrations failed to improve the uniformity of the coating color. The same negative results followed initial sealcoating of the tablets using shellac.

After varying dye concentrations and application techniques it was found that, per 10,000 commercial placebos (Pa), 50 ml. of Z-15-(T20)-3 containing 0.03% FD & C Red No. 3, added in a single continuous fine stream with twenty seconds of forced warm air applied immediately after all the tablets have been wetted by the coating fluid, produced the most desirable color. It is worth noting however, that the use of colored zein fluids, when applied in the same manner as the uncolored zein fluids, but with increasing concentrations of dye per application, can also result in a rather unique, strongly variegated coating, should this be desirable.

Visual Examination of Coated Tablets.—Random samples of the coated and uncoated tablets listed in Table II were examined for luster and homogeneity in the following manner. Evaluation of luster was performed in a darkened room using the reflectance of light from a 3-watt bulb on the 5× Aplanat Magnifier. The lens of the magnifier was removed for this inspection. The tablets were rated

TABLE I.—FORMULATIONS AND METHODS OF APPLICATION

Formula Number	Initial Tablets and Quantity Used	Blower Time[a]	Intervals[b]	Volume per Application, cc 1	2	3	4	5	6	7	8	9	10	11	12	Total
Z-15-(T20)-3 F D & C Red No. 3—0.03%	Pa—10M	20	...	50	...	...	...	...	...	...	...	...	...	...	...	50
Z-15-(T20)-3	Ax—10M	20	2	20	20	15	15	10	...	...	...	...	...	...	...	80
Z-15-(A83)-3	Px—10M	20	2	20	20	15	15	15	15	10	10	15	...	...	...	135
Z-15-(A83)-3	Ax—10M	20	3	20	20	15	15	15	15	10	10	15	...	...	...	100
Z-15	Pa—10M	20	2	20	15	15	10	10	15	...	...	...	...	...	...	85
Z-15-(S20)-3	Px—10M	20	1	20	10	10	10	10	10	10	10	...	...	...	...	80
Z-15-(S80)-3	Px—10M	30	1	20	10	10	10	10	10	10	10	...	10	10	10	90
Z-20-(T20)-1	Px—10M	20	2	20	20	15	10	15	10	10	10	10	10	10	10	150
Z-20-(T20)-5	Ax—8M	20	2	15	15	15	10	10	10	15	10	10	...	...	...	110
Z-20-(T20)-10	Px—10M	20	2	15	10	15	15	15	15	15	...	...	...	...	...	100
Z-15-(T80)-3	Px—6M	30	1	20	10	10	10	10	10	10	...	...	...	...	...	80
Z-5-(T20)-3	Px—10M	20	2	20	20	20	20	30	20	20	20	30	...	...	...	200
Z-10-(T20)-3	Px—8M	20	2	20	20	20	20	20	20	10	20	20	10	...	...	180
Z-20-(T20)-3	Px—8M	20	2	20	20	20	20	20	20	10	20	20	10	10	10	150
Z-15-(T20)-1	Px—10M	20	2	20	15	15	10	15	10	10	10	10	...	...	...	115
Z-15-(T20)-5	Px—10M	20	2	20	15	15	15	10	10	10	10	...	15	...	...	105
Z-15-(T20)-10	Px—10M	20	2	20	15	15	15	10	10	15	15	15	15	...	...	145

[a] Time in seconds during which forced warm air is directed on the tablets immediately after the coating fluid is evenly distributed.
[b] Time interval in minutes between applications of coating fluid.

TABLE II.—VISUAL EXAMINATION OF TABLETS

Formula Number	Initial Tablet	Luster[a]	Homogeneity[b]
Z-15-(T20)-3 FD & C Red No. 3–0.03%	Pa	2	4
Z-15-(T20)-3 waxed FD & C Red No. 3–0.03%	Pa	3	4
Z-15-(T20)-3	Ax	1	2
Z-15-(T20)-3 waxed	Ax	2	2
Z-15-(A83)-3	Px	3	2
Z-15-(A83)-3 waxed	Px	3	2
Z-15-(A83)-3 waxed	Ax	3	1
Z-15	Pa	2	3
Z-15 waxed	Pa	4	3
Z-15-(S20)-3	Px	4	4
Z-15-(S80)-3	Px	4	4
Ferrous sulfate	...	4	4
Sodium salicylate	...	4	4
Ammonium chloride	...	4	4
Cascara sagrada	...	4	4
Ferrous gluconate	...	4	4
Uncoated Pa	...	4	4
Uncoated Px	...	3	3
Uncoated Ax	...	0	0

[a] Based on commercial placebo (4) as standard. Decrease in number represents decrease in luster.
[b] Based on commercial placebo (4) as standard. Decrease in number represents decrease in homogeneity.

numerically for luster on a scale whereby the untreated commercial placebos (which possessed a very satisfactory luster without any coating) were rated as 4 and the untreated aspirin tablets having no luster rated as 0.

Homogeneity of the tablet surfaces was determined in a darkened room using the 5X Aplanat magnifying lens, the light source being the 3-watt bulb in the magnifier. Evaluation of homogeneity was numerical on a scale whereby untreated commercial placebos were rated as 4 (these tabets being very smooth and homogeneous in appearance). Values of 3 and 2 were assigned to tablets with decreasing homogeneity and a value of 1 for those tablets exhibiting a definite lack of homogeneity.

Infrared Heat Test.—Six each of the coated and uncoated tablets listed in Table II were selected at random and placed on the base plate of the infrared heat lamp, seven inches from the two 250-watt infrared lamps. The tablets were inspected at time intervals of 5, 10, 15, and 30 minutes, and 1, 2, 4, and 8 hours. Results of this test strongly indicate that zein coated tablets are more resistant to heat than the commercial sugar coated tablets and generally show little deleterious effects from the temperature employed. Zein coatings failed to protect the aspirin, which melted in every instance. All the zein coatings employed were free from the scorching effects of the heat. Waxed and unwaxed Pa colored with FD & C Red No. 3 began to fade after one-half hour exposure.

Durability (Shaking) Test.—Four-ounce glass French square bottles were filled to one-half capacity with coated and uncoated tablets as listed in Table II. These bottles were placed on their sides in the shaker and inspected at scheduled intervals for a total of two hours. This test is such that the bottles are shaken lengthwise through a total distance of approximately one inch three hundred times per minute.

During each examination of the tablets any powder which may have accumulated in the bottles during the test was removed to facilitate a more advantageous inspection. Results of these examinations indicate that zein coated tablets are more resistant to physical trauma than either the uncoated tablets or the commercially sugar coated tablets. Waxed zein coatings are more subject to chipping than the same unwaxed coatings.

Humidity Test.—Random samples of the tablets listed in Table III were put in uncovered Petri dishes which were then placed in a 250-ml. glass desiccator from which the desiccant had been removed and replaced with one inch of water. The tablets on each dish were in a single layer, thereby exerting only their own weight on the glass and not being compressed from above by other tablets. The dishes were filled to approximately three-fourths capacity to allow the tablets to slide freely when they were tilted at an angle. The Petri dishes used and the quantity of tablets employed were such that a maximum of visual inspection was possible with a minimum of epidermal contact. The conditions employed permitted a free movement of moist air equally over every tablet contained in the glass desiccator jar. The desiccator jar was securely closed but the Petri dishes contained therein were uncovered. The tablets were examined periodically for adherence to the glass dish which would indicate a softening of the coating due to the high humidity of the desiccator jar. Adherence values were assigned as described in Table III.

U. S. P. Tablet Disintegration Test.—Disintegration tests were performed on coated and uncoated tablets using the prescribed apparatus (10). Six each of the tablets listed in Table IV were tested in five replicates giving a total of five average disintegration values for each type tablet used. The average values are recorded in Table IV for disintegration in distilled water and artificial gastric juice as employed by Huyck (11).

TABLE IV.—DISINTEGRATION TEST

Coating Formula	Initial Tablet	Disintegration Time, Sec. Distilled Water	Disintegration Time, Sec. Artificial Gastric Juice
Z-15-(T20)-3 FD & C Red No. 3-0.03%	Pa	46	39
Z-15-(T20)-3 waxed FDC & Red 3-0.03%	Pa	47	39
Z-15-(T20)-3	Ax	22	20
Z-15-(T20)-3 waxed	Ax	27	23
Z-15-(A83)-3	Px	98	93
Z-15-(A83)-3 waxed	Px	116	102
Z-15-(A83)-3 waxed	Ax	32	30
Z-15	Pa	59	45
Z-15 waxed	Pa	63	44
Z-15-(S20)-3	Px	105	93
Z-15-(S80)-3	Px	119	106
Uncoated Pa	..	49	39
Uncoated Px	..	108	106
Uncoated Ax	..	12	12

DISCUSSION

Varying concentrations of zein (5 to 20) in 91% isopropyl alcohol were employed as nonenteric tablet coatings. In most instances additives were combined with these fluids. The type and quantity of additives employed varied and ranged in concentration from 1 to 10%. Certain of these tablet coating formulas were chosen for testing based entirely upon the general appearance of the finished coated tablet. Fluids containing 5% and 10% zein were too thin, requiring larger quantities to cover the tablets adequately. Furthermore, the tablets are thus exposed to more zein solvent, thereby permitting possible tablet disintegration in the coating pan. Zein concentrations of 20% resulted in rough, uneven coatings. Additive concentrations of 10% resulted in a greatly prolonged drying time. Fluids containing 15% zein and 3% additive were sub-

TABLE III.—HUMIDITY TEST

Formula Number	Initial Tablet	Time, in Days[a] 1	2	3	4	7	8	9	11	14
Z-15-(T20)-3 F D & C Red No. 3-0.03%	Pa	0	0	0	0	0	0	0	0	0
Z-15-(T20)-3 waxed FD & C Red No. 3-0.03%	Pa	0	0	0	0	0	0	0	0	0
Z-15-(T20)-3	Ax	4	6	6	8	6	6	6	6	6
Z-15-(T20)-3 waxed	Ax	3	3	4	4	3	3	3	4	6
Z-15-(A83)-3	Px	0	0	0	0	0	0	0	0	0
Z-15-(A83)-3 waxed	Px	0	0	1	1	1	1	0	2	2
Z-15-(A83)-3 waxed	Ax	0	0	2	3	3	4	4	5	6
Z-15	Pa	0	0	0	0	0	0	0	0	0
Z-15 waxed	Pa	0	0	2	2	1	0	0	2	0
Z-15-(S20)-3	Px	0	0	0	0	0	0	0	0	1
Z-15-(S80)-3	Px	0	0	0	0	0	0	0	0	0
Ferrous sulfate	..	4	7	8	8	10	10	10	10	10
Sodium salicylate	..	3	3	3	5	8	9	10	10	10
Ammonium chloride	..	3	4	5	9	10	10	10	10	10
Cascara sagrada	..	3	4	6	8	10	10	10	10	10
Ferrous gluconate	..	2	3	3	3	8	10	10	10	10
Uncoated Pa	..	0	0	0	0	0	0	0	0	0
Uncoated Px	..	0	0	0	0	0	0	0	0	0
Uncoated Ax	..	0	0	0	0	0	0	0	0	0

[a] 0 adherence—all tablets slide readily when dish is tilted; 3 adherence—tablets slide only when dish is gently tapped with finger; 10 adherence—none of the tablets were released when dish is tapped as above; all required individual pressure to move them, leaving a portion of the tablet and coating remaining on the glass.

mitted to testing because these concentrations appeared most efficacious with respect to uniformity and ease of coating. Total working time employing the prescribed concentrations of zein and additive was less than one-half hour for the actual coating procedure. At the end of this period the coating was dry and but for a characteristic odor of zein and isopropanol, the tablets were ready for packaging. This odor gradually dissipates when the tablets are exposed to air. The need for sealcoating, cooking kettles, and ovens is eliminated and the size and weight of the coated tablets is practically unchanged (1–5 mg. weight gain). Zein coated tablets resisted effects of the durability, humidity and infrared heat tests to a greater degree than did the commercial sugar coated tablets. It would appear that aspirin presents an individual problem. Zein coatings completely mask the characteristic odor of acetic acid ordinarily emitted from aspirin tablets and remain on these tablets long enough to prevent the very familiar bitter taste of this drug from being experienced by the subject. Disintegration time is but slightly effected by the zein coatings and emblems, trademarks or grooves on a tablet surface are clearly visible through the coating.

From the design of this work it can be seen that no comparison between individual zein coating formulas was intended and that no such evaluations can be made from the accumulated data. It does appear, however, that zein can be well utilized as a nonenteric tablet coating material when applied in the form of a thin film.

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Book Notices

Methoden der organischen Chemie (Houben-Weyl), 4th ed. Vol. XI/1. Edited by EUGENE MÜLLER. George Thieme Verlag, Stuttgart, 1957. Price $48.50 (subscription price $44.55).

In Volume XI of this handbook, the methods of preparation and transformation of amines, lactams, quaternary ammonium compounds, and nitrogen compounds containing sulfur are discussed. Methods for the direct introduction of amino groups including methods involving addition reactions, reduction, condensation, the use of organometallic compounds, rearrangement, fission, and other special procedures are discussed quite in detail. Preparative methods for primary, secondary, and tertiary amines are described in a separate chapter and the properties and manipulation of ammonia are also discussed in another chapter dealing with this subject. Volume XI/1 is of the same high type of binding and format as the earlier volumes in this series.

Biochemistry of Some Peptide and Steroid Antibiotics. By E. P. Abraham. John Wiley & Sons, Inc., New York, 1957. ix + 96 pp. 12.5 x 18.5 cm. Price $3.

This compilation of the 1957 Ciba Lectures in Microbial Biochemistry, at Rutgers University, by Dr. Abraham (Oxford), describes recent work on the bacitracins, the cephalosporins, and their structural and functional relationships to other antibiotics.

Unexpected Reactions to Modern Therapeutics. Antibiotics. By Leo Schindel. Charles C Thomas, Springfield, Ill., 1957. xii + 146 pp. 12.5 x 18.5 cm. Price $3.

This survey deals first with unexpected reactions to antibiotics, describing signs and symptoms due to these reactions. The antibiotics discussed are: penicillin, streptomycin-dihydrostreptomycin, chloramphenicol, tetracyclines, antibiotic-resistant bacteria, neomycin, erythromycin, bacitracin, fumagillin, novobiocin, cycloserine, and polymyxin. The management of unexpected reactions is considered. References are given at the end of each chapter, and a general subject index is appended

Atomic Energy in Agriculture. By William E. Dick. Philosophical Library, New York, 1957. 13.5 x 22 cm. Price $6.

This book describes how atomic energy may speed up plant breeding and the production of new varieties of plants, and how atomic energy can be used in other ways in agriculture.

Dental Practitioners' Formulary 1957. By the Joint Formulary Committee. The British Medical Association, London, 1957. 49 pp. 10 x 16 cm. Price 3s.

This third edition of the Dental Practitioners' Formulary is intended for use by dentists in the British National Health Service.

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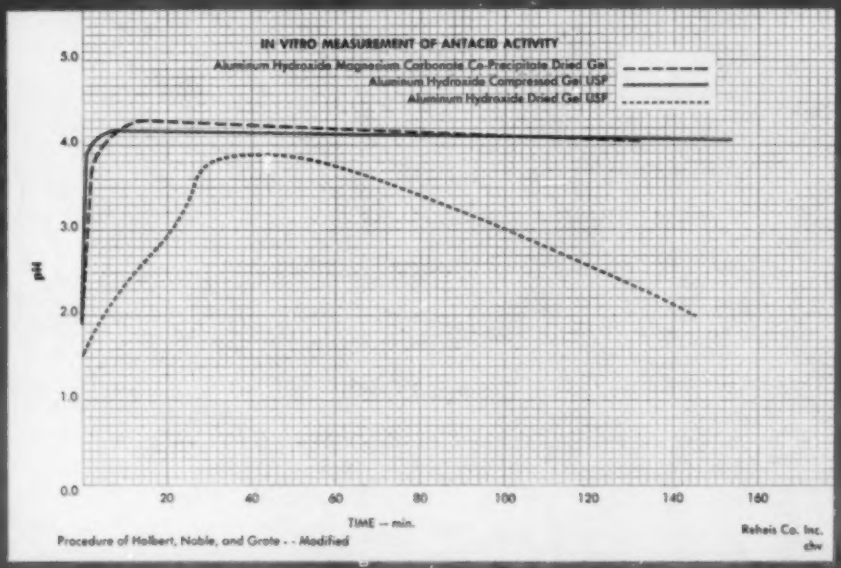

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